

FRDC FINAL REPORT

ASSESSMENT OF FISH GROWTH PERFORMANCE UNDER LIMITING ENVIRONMENTAL CONDITIONS: AQUACULTURE NUTRITION SUBPROGRAM

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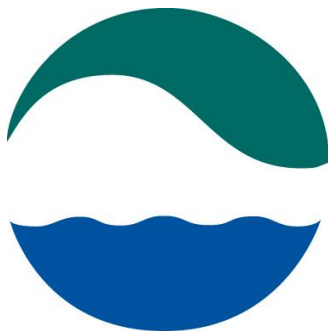
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Non-technical Summary

2004/237 ASSESSMENT OF FISH GROWTH PERFORMANCE UNDER LIMITING ENVIRONMENTAL CONDITIONS: AQUACULTURE NUTRITION SUBPROGRAM

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OBJECTIVES

1. Generate critically identified data for incorporation into factorial growth models that describe nutrient supply and nutrient retention in relation to temperature.
2. Determine the maintenance dietary protein and energy requirements for Atlantic salmon at normal to high temperatures.
3. Develop biochemical tools to understand the effect of temperature on the growth performance of fish.
4. Determine the optimum dietary protein to energy ratio for Atlantic salmon at normal to high temperatures.
5. Use biochemical tools to understand the effect of temperature on protein turnover and growth performance in barramundi.
6. Use biochemical tools to understand the effect of temperature on protein turnover and growth performance in Atlantic salmon.

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED TO DATE

Australian aquafeed manufacturers have increased information available for formulation of feeds or to assist in feed management on farms.

Greater fundamental knowledge about the physiological mechanisms that explain how fish maintain growth under limiting environmental conditions.

Enhanced capacity to understand and quantify the nutritional, physiological and molecular effects of limiting environmental conditions on Australian fish species.

Increased capacity for strategic and applied research related to environment and nutrition and to respond to global issues such as climate change.

Increased technical knowledge in industry through industry presentations, training of research students and inclusion of material in courses.

This research has advanced our understanding of how fish growth is influenced by nutrition, by environment and by the interaction between nutritional and environmental factors. When the research started the majority of nutrition research considered the performance of feeds under optimum environmental conditions. In stark contrast the Australian aquaculture industry is increasingly facing the proposition of growing fish under sub-optimum conditions. Atlantic salmon are grown at elevated summer temperatures whereas barramundi and several sub-

tropical species are affected by low winter temperatures. In addition, aquafeed companies lacked critical information about the optimum balance of protein and energy required for feed formulations at elevated temperatures. The research addressed a significant need for fundamental and applied information about nutrition of fish under limiting environment conditions. Integration of molecular techniques into the research program enhanced the value of the research considerably.

We successfully modelled protein and energy requirements for seawater Atlantic salmon at an elevated temperature of 19°C. This temperature was selected because although it is at the upper end of temperatures at which feeding would continue on-farm, salmon still feed and grow. Importantly, Atlantic salmon exhibited the same type of response to differences in dietary composition and nutrient intake as at lower temperatures. The research provided critical and previously unknown information for aquafeed companies to use for their high temperature feed formulations. For example, the optimum dietary protein to energy for Atlantic salmon diets fed at 19°C was predicted to be 19.8 grams of digestible protein per MJ of energy.

A critical feature of elevated temperature is decreased oxygen content in water. A second experiment compared the performance of seawater Atlantic salmon at 19°C at moderately low dissolved oxygen typical of those experienced in summer on-farm. Overall, growth performance was significantly affected by oxygen but only marginally by diet composition. Low oxygen decreased appetite and caused lower growth, growth efficiency was not affected.

Two barramundi growth trials were used to model the effects of temperature on the feed intake and growth performance across a wide temperature range from 21 to 39°C. The optimal temperature for growth of juvenile barramundi was 31°C. Feed intake, SGR and growth efficiency remained at ≥90% of the maximum biological response over a large temperature range of 8°C. These models showed that juvenile barramundi are eurythermal and therefore maximise growth by adopting strategies to optimise nutrient utilisation over as wide a temperature range as possible. So although farming often uses temperatures which are above and below the optimum high performance still occurs. The decrease in growth efficiency is rapid at extreme (high) temperature and occurs over only a few degrees, this was investigated to understand the upper thermal tolerance of barramundi.

An important achievement of the project was to understand potential uses of biochemical and molecular tools in nutrition research. To do this, the utilisation and fates of dietary protein were measured in different ways. Protein synthesis was measured in barramundi across a broad temperature range. Protein synthesis was not significantly different over the optimum temperature range and, more critically, synthesis retention efficiency was highest over this range. Protein is expensive to make and it is retained most efficiently during optimum growth. Nitrogenous excretion was measured in Atlantic salmon and related to dietary composition and feed intake. Free pool amino acid concentrations in muscle were measured in relation to temperature and starvation to investigate their potential to highlight limiting nutrients. Starvation had a greater effect on free pool amino acid concentrations than temperature, the essential amino acid lysine had a very low concentration which suggested it may be a limiting amino acid. Importantly, the data for the initial group suggested that in normally feeding Atlantic salmon the dietary amino acids were not limiting at an elevated temperature.

Changes in expression of genes involved in protein turnover were assessed to investigate their potential as indicators of responses to nutritional and environmental variables. Starvation resulted in significantly higher expression of salmon muscle cathepsin L and proteasome β subunit N3. In barramundi cathepsin D was responsive to differences in nutritional status of feeding fish held at an elevated temperature. By combining molecular and biochemical approaches a mechanism for coping with high temperatures was proposed, a greater energy requirement was met by amino acids supplied by both the diet and muscle protein breakdown via specific pathways. This explained why high protein diets work at high temperatures.

KEYWORDS: Aquafeeds, Atlantic salmon, barramundi, elevated temperature, extreme environmental conditions, growth, nutrition.

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Background

Understanding the interaction between fish nutrition and environmental parameters has an extremely high priority in several sectors of the Australian finfish aquaculture industry. Climate change appears to be causing high summer water temperatures in salmon growing regions. Barramundi are being farmed under an increasingly diverse range of systems. Generally, fish growth performance is being pushed harder. Although aquafeeds have changed dramatically there is a real need to match formulations more accurately to environmental conditions, particularly temperature. This is because aquafeeds are formulated to more closely meet the actual nutritional requirements and therefore have lower “margins for error”; they now contain a diverse range of ingredient sources that introduce different sets of positive and negative characteristics; they are a major production cost and major sources of environmental loading. Limiting environmental conditions, particularly those imposed by extreme temperatures that are outside of the optimum range, will test the adequacy of nutrient supply and the efficiency of growth. It is important to note that research into aquaculture nutrition often accumulates data under optimum experimental conditions that maximise growth performance. There is a need to understand growth under more extreme conditions. The research proposed here aims to test and model growth performance under the most relevant limiting conditions and to develop and assess the use of biochemical tools used to understand growth performance.

This application was submitted through TasFRAB and was given in principle support to progress through the Atlantic Salmon Aquaculture and Aquaculture Nutrition (ANS) Sub-programs. The FRDC Programs Leader advised that it should address generic research and industry issues and consequently it was submitted through the ANS. This research is applicable to all aquaculture species and focuses on using two major species as models for cold-water and tropical fish species. In addition, in principle support was gained for the project at the annual ANS workshop held in Fremantle (29-30th May 2003).

Skretting were a major partner in the proposed research. This was of considerable relevance to the transfer of results to both the barramundi and salmon farming industry. At the time of the application Skretting manufactured and supplied the majority of aquafeeds for both of these species as farmed in Australia as well as being the drivers behind Australia's largest barramundi venture (Marine Harvest NT).

Need

The research proposed here has received consistently strong support from the TSGA (Tasmanian Salmonid Growers Association), the TasFRAB, the Atlantic Salmon Aquaculture Sub-program and the Aquaculture Nutrition Sub-program. The research addresses two challenges in the FRDC R&D Plan 2000-2005:

Challenge 2. Increasing production through aquaculture (FRDC R&D Plan 2000-2005) is addressed by this research. The research proposed contributes by increasing understanding of how to increase production under Australian environmental conditions and how to increase efficiency.

Challenge 5. Reducing the quality of fish protein fed to aquatic livestock (FRDC R&D

Plan 2000-2005). The proposed research contributes by increasing understanding of processes that drive growth efficiency.

It must be stressed that the research proposed here has been developed in collaboration with Skretting and this means that results will impact on aquafeeds rapidly and in a meaningful way. However, the data generated will be disseminated for use by other feed manufacturers, the aquaculture industry and other researchers.

Tactical Needs

The proposed research aims to demonstrate which feeds are most appropriate for use at the different temperatures experienced by both the salmon and barramundi aquaculture industry during the normal production cycles and over the geographical range they are farmed. The research has to be conducted in Australia because the performance of Atlantic salmon at high temperatures is not important in Northern Europe and the performance of barramundi at low temperatures is not relevant in South East Asia. Consequently, it is unlikely that commercially driven research of relevance to Australian conditions will be conducted outside of Australia. The vital importance of understanding the relationship between dietary protein and energy in relation to temperature and low growth has been very clearly identified by individual salmonid growers and by the TSGA for several years. However, farm data cannot be used to solve the issues retrospectively and a clear need for controlled experiments exists. Barramundi aquaculture is expanding rapidly across Australia and they are being farmed under many different environmental (temperature) regimes, there is a clear need to understand the relationship between dietary protein and energy in relation to temperature. In addition to providing direct information about the performance of both species of fish in relation to diet composition the experiments are designed so that data can be incorporated into "factorial models" of growth. These are used by feed companies as well as under development by key researchers. The proposed research will make an important contribution to the database for these and for both species.

Strategic Needs

The research seeks to address the lack of information about mechanisms that determine efficient growth in fish by consideration of key physiological and nutritional variables in relation to temperature. The PI has an extensive record of using physiological tools to investigate amino acid (protein) and energy utilisation by fishes. Developing this approach in relation to temperature is of strategic importance for equipping Australian aquaculture with tools to investigate other species. Protein synthesis is a major driver of growth but it is energetically expensive (20-40% of costs of growth) and understanding the interaction between diet composition and efficient growth is a major driver behind the proposed research. The research will develop physiological tools using barramundi as a model, these will then be tested and developed further using salmon.

Objectives

1. Generate critically identified data for incorporation into factorial growth models that describe nutrient supply and nutrient retention in relation to temperature.
2. Determine the maintenance dietary protein and energy requirements for Atlantic salmon at normal to high temperatures.
3. Develop biochemical tools to understand the effect of temperature on the growth performance of fish.
4. Determine the optimum dietary protein to energy ratio for Atlantic salmon at normal to high temperatures.
5. Use biochemical tools to understand the effect of temperature on protein turnover and growth performance in barramundi.
6. Use biochemical tools to understand the effect of temperature on protein turnover and growth performance in Atlantic salmon.

Research

Chapter 1.

Biochemical tools for assessment of growth performance in fish

Chapter 2.

Optimum dietary protein to energy ratio for seawater Atlantic salmon, *Salmo salar* L., at an elevated temperature of 19°C

Chapter 3.

Influence of dissolved oxygen on the optimum dietary protein to energy ratio for seawater Atlantic salmon, *Salmo salar* L., at an elevated temperature of 19°C

Chapter 4.

Maintenance requirements for seawater Atlantic salmon, *Salmo salar* L., at different temperatures

Chapter 5.

Assessment of possible temperature dependent limiting nutrients in seawater Atlantic salmon, *Salmo salar* L., at two temperatures

Chapter 6.

Assessment of growth performance and protein turnover of individual seawater Atlantic salmon, *Salmo salar* L., at an elevated temperature of 19°C

Chapter 7.

The effect of temperature on growth performance in barramundi, *Lates calcarifer* (Bloch)

Chapter 8.

The effect of temperature on protein synthesis in barramundi, *Lates calcarifer* (Bloch)

Chapter 9.

Growth performance and protein turnover in barramundi, *Lates calcarifer* (Bloch), fed two dietary protein levels at three temperatures

Chapter 1

Biochemical tools for assessment of growth performance in fish

Abstract

Understanding how dietary protein is retained provides the key to understanding nutrient utilisation and growth performance of fish. The supply of energy and the use of non-protein energy underlie the efficient use of protein for growth. Use of a factorial approach to model protein and energy requirements is introduced and links between utilisation of energy and protein and protein metabolism are made through an expanded protein-nitrogen flux model that incorporates protein turnover through protein synthesis and protein degradation. The measurement of protein synthesis and of protein degradation pathways, using gene expression, is outlined. These models are used in order to provide a fundamental approach to understanding growth performance in relation to extreme environmental conditions. A major aim of the research was to understand changes in growth over a wide temperature spectrum using barramundi as a model species. The focus on salmon was different and explored effects of an elevated temperature that was at the margins of where the species can be grown. Models also allowed the potential of biochemical tools to be assessed by understanding their role in growth. This chapter provides an overview of the approaches and methods that were used in the research that is presented in the remainder of the report.

1.1. Introduction

Understanding fish growth (Brown, 1957; Brett, 1979; Elliott, 1979), particularly under commercial production (Cho et al., 1982; Hephher, 1988; Bureau et al., 2002; Bar et al., 2007), is clearly important and has been the focus of a considerable amount of research, review and debate. Major drivers of research are being able to model and then predict performance based on responses to a set of known variables and being able to understand the biological basis of performance in order to improve it. This chapter concerns the approaches taken and the development of the methods that were used to assess and understand growth performance of Atlantic salmon (*Salmo salar* L.) and barramundi (*Lates calcarifer* (Bloch)) when subject to different environmental and nutritional regimes. The principle focus with Atlantic salmon was to use dietary manipulation to better understand protein and energy requirements at an elevated temperature. For barramundi, the principle aim was to better understand growth performance in relation to the effect of temperature across the range experienced commercially, from low to elevated temperature. In order to do this, traditional methods based on nutrient intake and changes in carcass composition were considered in conjunction with factorial modelling, measurement of protein-nitrogen flux and changes in expression of specific genes.

1.1.1. Carcass composition and factorial modelling

Methods for chemical analysis of whole body chemical composition have been conducted on production animals for many years and are routinely used in virtually all published research on fish nutrition. To be accurate proximate analysis, which gives composition as moisture, ash, crude protein, ether extract, crude fibre and nitrogen-free extractives composition (McDonald, 1988), is not often performed on fish. Chemical analysis is most often provided for moisture, ash, crude protein and crude lipid / fat;

gross energy is measured in less than half of recent publications and carbohydrates are rarely measured in fish.

Recently there has been renewed interest in the use of factorial modelling of fish growth. The majority of research has focused on protein and energy requirements (Shearer, 1995; Lupatsch et al., 2001a; Lupatsch et al., 2001b; Azevedo et al., 2005; Bureau et al., 2006) although the approach has also been adapted to determine amino acid requirements (Hauler and Carter, 2001a; b; Hauler et al., 2007) and other nutrients (Shearer, 1995). The key components involve determination of metabolic weight exponents, digestibility, maintenance requirements and efficiency of retention for both protein and energy. The application of this approach and determination of key components is the subject of Chapters 2 and 3 where there is further discussion.

1.1.2. Protein-nitrogen flux

The utilisation of dietary protein is one of the major drivers of growth performance (Boorman, 1980; Lupatsch et al., 2001a; Azevedo et al., 2005). It is well recognised that both the quantity and quality of protein intake significantly influence growth and growth efficiency. High protein intake above the requirement results in lower protein retention efficiency and higher excretion of metabolic nitrogenous waste. Poor protein quality, as reflected by an imbalanced dietary amino acid profile, will have the same effect: lower protein retention efficiency and higher nitrogenous excretion (Carter and Houlihan, 2001). Protein-nitrogen budgets describe protein-nitrogen flux and how consumed protein-nitrogen (C_N) is either lost as faecal nitrogen waste (F_N), excreted as metabolic nitrogen (U_N) or retained as production (P_N) so that $C_N = F_N + U_N + P_N$ or $P_N = C_N - (F_N + U_N)$ (Carter and Brafield, 1991; Carter and Brafield, 1992). These are useful in themselves and gain further value from inclusion of metabolic pathways (Fig. 1.1), protein synthesis and degradation for example (Carter et al., 1998).

Protein synthesis is the complex process through which amino acids are combined into proteins, at the same time proteins undergo degradation into amino acids. The synthesis and breakdown of protein is termed protein turnover and is thought to occur to allow damaged proteins to be replaced so that they do not accumulate and disrupt important physiological functions (Waterlow, 1999). Protein synthesis and protein degradation can be incorporated into protein-nitrogen budgets (Carter et al., 1998). This provides an opportunity to understand the processes that influence the synthesis and retention of proteins. For example, amino acids will only be used for protein synthesis in the proportion they are present in the many proteins that make up the fish. Where there is an excess or imbalance of amino acids they will be used for energy, and the remaining nitrogen (amine group) excreted and not retained for growth. Of further interest is that fish appear to use protein synthesis to regulate the influx of amino acids following a meal. Thus, quite different dietary composition may stimulate protein synthesis to a similar extent but for different reasons (Young et al., 1991; Carter and Houlihan, 2001). In the case of a well balanced diet, protein retention will be high and the efficiency of retaining synthesised protein high. In contrast protein retention will be low on a poor diet, if the diet stimulated high protein synthesis the efficiency of retaining synthesised protein will be low and protein degradation high. It is also possible that a poor diet may not stimulate high protein synthesis, in which case protein degradation would not be high either. However, a poor diet would increase

nitrogenous excretion regardless of whether protein synthesis and degradation were stimulated.

Protein synthesis is energetically expensive and can use around 20 to 40% of the total energy used by fast growing juvenile fish (Carter and Houlihan, 2001). This is not problematic if growth is high since the majority of “expensive” protein is retained as growth. With low growth and high protein synthesis on a poor diet fish regulate the concentration of imbalanced amino acids through protein synthesis and short term retention, the “wasted” energy is obviously not then available to fuel long-term growth.

Whilst the measurement of protein synthesis has been achieved using several methods (Houlihan et al., 1986; Carter et al., 1994; Houlihan et al., 1995) the direct measurement of protein degradation at a physiological level remains elusive due to the need to measure a more complex set of biochemical pathways. Currently protein degradation is calculated as the difference between protein synthesis and protein retention (Weisner and Zak, 1991), a useful but unsatisfactory value since it is not independent of the other key variables of protein turnover. Quantitatively the major routes are via the autophagic-lysosomal and the ubiquitin-proteasome systems, with calpains as a lesser pathway (Attaix et al., 1999). It is now possible to assess the value of using molecular methods to measure protein degradation, the focus is to determine whether there are changes in the expression of known genes involved in protein degradation pathways and whether these can be related to changes in growth at a whole animal level (Martin et al., 2001; Martin et al., 2003; Dobly et al., 2004).

1.1.3. Gene expression

Transcriptional mechanisms of protein degradation associated with nutritional restriction, fasting, cancer, diabetes, sepsis and muscle denervation are well characterised in mammalian models. More recently similar approaches have been applied in a limited number of studies of fish in an attempt to characterise the transcriptional response of known genes to a variety of conditions associated with muscle atrophy (Martin et al., 2001; Martin et al., 2002; Dobly et al., 2004; Mommsen, 2004; Salem et al., 2006a; Salem et al., 2006b; Salem et al., 2007). The major proteolytic pathways in fish are similar to those of mammals, as previously mentioned; however, recent studies indicate that the ubiquitin-proteasome pathway, known to dominate the degradation of muscle protein in mammals, has a greatly reduced role in fish muscle proteolysis. Furthermore, it seems that the lysosomal proteolytic pathway, incorporating the cathepsin group of proteases, or the calpain pathway is the major proteolytic pathway in fish muscle. Whilst the relative contribution of the major proteolytic pathways to protein degradation of muscle may be different it appears that, as in mammals, the expression of fish proteolytic genes is tightly controlled at the transcriptional level. It is therefore envisaged that using quantitative real-time PCR to determine the relative mRNA abundance of specific proteolytic genes allows a highly sensitive measurement of protein degradation in fish and may be used to compliment the determination of protein turnover.

1.1.4. Correlates of growth performance

From the above discussion it is apparent that several measurements can be used singularly or in combination to provide information about the physiological and nutritional status of fish under different dietary and environmental regimes. The approach taken in the present study was to relate these to dietary protein and energy utilisation through consideration of protein-nitrogen flux and protein turnover combined with factorial growth models. The aim was to assess the use of the different measurements and to provide an approach to studying the effects of diet and temperature on fish performance.

1.2. Materials and methods

1.2.1. General chemical analysis

Standard methods were used for chemical analyses of fish carcass, feeds and faeces: dry matter; nitrogen (Kjeldahl using a selenium catalyst); crude fat (Bligh and Dyer, 1959); gross energy (bomb calorimeter: Gallenkamp Autobomb, calibrated with benzoic acid), ash (AOAC, 1995). Similarly, validated techniques were used for other analysis including chromium (Furukawa and Tsukahara, 1966), yttrium and ytterbium (Ward et al., 2005).

1.2.2. Tissue composition

Protein and RNA

Protein concentration was measured using a modification of the folin-phenol method (Lowry et al., 1951) and RNA concentration was measured using dual wavelength absorbance (Ashford and Pain, 1986). Protein growth was calculated from changes in whole body protein as $k_g = 100 \cdot ((\ln P_2 - \ln P_1)/t)$, where P_1 and P_2 were the initial and final protein content of individual fish and t the time in days. The mean protein content of initial groups was used to calculate the initial protein content (P_1) of individual fish based on their wet weight.

Ninhydrin

Ninhydrin-positive substances (NPS) were measured according to established protocols (Shumway et al., 1977). Approximately 100 mg frozen muscle tissue was weighed and homogenized in 6 ml 80% ethanol. After centrifugation (10 min, 4°C, 5500 g) 50 µl of supernatant were removed and 950 µl of distilled water added to 4 ml of ninhydrin solution and 2 ml of hydrazine sulphate solution. Once the ninhydrin solution has been added the tubes were kept dark, heated at 100 °C for 10 minutes and cooled in air and then cold water. Leucine standards were made up in the range 0 – 600 µg ml⁻¹ (0 – 4.58 µM NPS ml⁻¹) in 80% ethanol. The optical density was determined at 570 nm and results were expressed as µM NPS g⁻¹ wet tissue.

Free amino acids

Approximately 200 mg of white muscle tissue was weighed into a tared glass test tube and homogenized in 4 ml of absolute ethanol plus 0.1 ml norvaline (10 µmol ml⁻¹) as an internal standard, and 0.9 ml distilled water. The homogenate was centrifuged (10 min, 4°C, 6000 g) and a 50 µl sub-sample of supernatant transferred to a microcentrifuge tube and freeze dried. Analysis was exactly according to previous

methods (Lyndon et al., 1993; Carter et al., 2000). The freeze dried samples were sent to Australian Protein Analysis Facility (APAF). In present study, tryptophan and arginine were not reported because tryptophan was too low and arginine was co-eluting with a contaminant peak found in the blank.

1.2.3. Protein-nitrogen flux and protein turnover

Nitrogenous excretion

The total ammonia present in the water samples was determined using the salicylate-hypochlorite assay. Urea concentration was determined by treating samples with urease followed by the salicylate-hypochlorite assay (Chapter 6).

Protein synthesis by flooding dose

Rates of protein synthesis were measured following a single injection of ^3H -phenylalanine using the flooding-dose method (Garlick et al., 1980; Houlihan et al., 1995). This method has been validated for use with both barramundi (Katersky and Carter, 2007) and Atlantic salmon (Carter et al., 1993b). In this report it was used with barramundi only. Twenty-four hours after the last meal fish were injected via the caudal vein with injection solution at rates of approximately $1.0 \text{ ml } 100 \text{ g}^{-1}$. Following injection, fish were then returned to separate aquaria containing aerated water. The injection solution contained $150 \text{ mmol L-phenylalanine}$ and $\text{L-(2,6-}^3\text{H) phenylalanine}$ (Amersham Pharmacia Biotech, NSW, Australia) in $0.2 \mu\text{m}$ filtered seawater at pH 7.4. The measured specific activity of the injection solutions were about 1100 (1123 ± 118) dpm nmol^{-1} phenylalanine. Incorporation times were varied between 60 and 130 min.

The treatment of samples to measure protein-bound and free-pool phenylalanine-specific radioactivity was as described previously (Houlihan et al., 1988; Carter et al., 1993a). Fractional rates of protein synthesis (k_s : $\% \text{ d}^{-1}$) were calculated as $k_s = 100 \cdot ((S_b/S_a) \cdot (1440/t_1))$, where S_b is the protein-bound phenylalanine-specific radioactivity at time t_1 (min) and S_a the free-pool phenylalanine-specific radioactivity (Garlick et al., 1980). Protein and RNA concentration were measured as above. Fractional rates of protein degradation (k_d , $\% \text{ d}^{-1}$) were calculated as $k_d = k_s - k_g$, (Millward et al., 1975). RNA was also expressed as the capacity for protein synthesis (C_s : $\text{mg RNA} \cdot \text{g protein}^{-1}$) and as RNA activity (k_{RNA} : $k_s \cdot \text{g}^{-1} \text{ RNA} \cdot \text{d}^{-1}$) (Sugden and Fuller, 1991).

1.2.4. Gene expression

Genes known to be involved in the three major proteolytic pathways were selected and their transcriptional products quantified using real-time reverse transcriptase PCR. However, relatively few barramundi genes have been isolated and submitted to Genbank. Similarly, the genes selected to represent the proteolytic pathways of Atlantic salmon were also unavailable from Genbank. Therefore the selected genes (Table 1.1) including both the proteolytic and housekeeping genes unavailable from Genbank for both barramundi and Atlantic salmon were initially isolated and at least partially characterised prior to quantification of their mRNA by real-time RT-PCR. Homology cloning using degenerate PCR primers designed from other fish species was used to obtain partial sequences of the selected genes. The design of primers for several of the selected Atlantic salmon genes was aided by EST contigs, identified as

partial sequences of the genes of interest, that were available from Atlantic salmon EST databases (SGP database <http://www.salmongenome.no> and GRASP database <http://web.uvic.ca/cbr/grasp>). Characterisation of the full length cathepsin D and L mRNA from barramundi was obtained using a SMARTTM RACE cDNA Amplification Kit (Clontech, USA) and were submitted to Genbank with the accession numbers EU143237 and EU143238, respectively.

RNA isolation and preparation

Total RNA was extracted from white muscle stored in an RNA preservation reagent (25 mM sodium citrate, 10 mM EDTA, 10M ammonium sulphate, pH 5.2) and purified using TRI Reagent[®] (Molecular Research Center, Cincinnati, OH, USA) including DNase treatment (DNA-freeTM, Ambion, Austin, TX, USA). RNA yield (A_{260}) and purity ($A_{260/230}$ and $A_{260/280}$) were determined spectrophotometrically and the integrity of the RNA was estimated from gel electrophoresis on a 1% agarose gel.

Reverse transcription

First strand cDNA was synthesised from total RNA (5 µg) using a SuperScript III First-Strand cDNA Synthesis kit (Invitrogen, VIC, Australia) with Oligo (dT)₂₀ priming according to the manufacturer's instructions. The reactions were incubated at 65°C for 5 min then 50°C for 50 min before the reverse transcriptase enzyme was inactivated at 85°C for 5 min. First strand cDNA reactions (21 µL) were diluted to 200 µL using nuclease-free water (Sigma-Aldrich, NSW, Australia) and stored at -20°C until quantitative PCR was performed.

Cloning and sequencing

Homology cloning using degenerate PCR primers was used to obtain partial sequences of the eukaryotic elongation factor 1 α (EF1 α), cathepsin D (CatD), cathepsin L (CatL) and β -proteasome subunit N3 (ProtN3) genes. Degenerate PCR primers were designed from multiple alignments of gene sequences from other fish species available in Genbank. PCR products were gel purified and cloned into pDrive vectors (Qiagen, VIC, Australia) followed by sequencing (AGRF, QLD, Australia). By assembling the sequences using SeqMan II (Lasergene v7.0, DNASTAR), partial sequences for genes of interest were obtained and identified. These sequences were either used directly to design primers for quantitative PCR or gene specific primers for both 5' and 3' RACE.

RACE (5' and 3' –rapid amplification of cDNA ends)

A SMARTTM RACE cDNA Amplification Kit (Clontech, USA) was used to isolate both CatD and CatL cDNA 3'- and 5'-ends. Firstly, the First-Stranded 3'-RACE-ready and 5'-RACE-ready cDNA samples from *L. calcarifer* were prepared according to the manufacturer's protocol (SMARTTM RACE DNA Amplification Kit, User Manual, Clontech, USA). The 3'-RACE-ready cDNA and 5'-RACE-ready cDNA were used as templates for 3'-RACE and 5'-RACE, respectively. CatD and CatL cDNA 3'-ends were amplified using 3'-gene specific primers and the universal primers provided by the kit. For the first PCR amplifications of 3'-RACE, CatD3-1 (5'-

AGACATCGCCTGCTTGCTTCATCACA-3') and CatL3-1 (5'-CGTGCTCTGGACTGGAGGGATAA GG GC-3') and UPM (Universal Primer Mix, provided by Clontech) were used as the first PCR primers (3'-RACE), and 3'-RACE-ready cDNA was used as a template. For the nested PCR amplification of 3'-RACE, CatD3-2 (5'-GAAGAACGGCACTGCCTTTGCCATCC-3') and CatL3-2 (5'-GCTACGTCCTCCCGTTAAAGACCAG-3') and NUP (Nested Universal Primer, provided by Clontech) were used as the nested PCR primers (3'-RACE), and the products of the first PCR amplification were used as templates. CatD and CatL cDNA 5'-ends were amplified using 5'-gene-specific primers and the universal primers provided by the kit. For the first PCR amplification of 5'-RACE, CatD5-1 (5'-CGAGGATCCCATCAAACCTGGCTGCG-3') and CatL5-1 (5'-GTTGCAGCCCTCGTTGCCCTCGGTC-3') and UPM were used as the first PCR primers (5'-RACE), and 5'-RACE-ready cDNA was used as templates. For the nested PCR amplification of 5'-RACE, CatD5-2 (5'-ACAGACATCGCCTGCTTCTTCA -3') and CatL5-2 (5'-GGAGCAGTCCACCAGGTTCTGTTCAC-3') and NUP were used as the nested PCR primers (5'-RACE) and the products of the first PCR amplification were used as templates. For the first and nested PCR amplification of CatD and CatL cDNA 3'- and 5'-ends, an AdvantageTM 2 PCR Kit (Clontech, United States) was used. The first and nested PCR procedures were carried out under the same conditions described in the protocol (SMARTTM RACE cDNA Amplification Kit, User Manual, CLONTECH. By 3'-RACE and 5'-RACE, both CatD and CatL ends were respectively obtained. The products were sub-cloned into pDrive vectors (Qiagen) followed by sequencing (AGRF). By assembling the sequences of 3'-RACE, 5'-RACE and the core fragment on SeqMan II (Lasergene v7.0), the full length cDNA sequences of CatD and CatL were obtained.

Quantitative PCR

Real-time PCR primers (Table 1.1) were designed from partial sequences obtained using degenerate PCR primers and from the full length cDNA sequences obtained using RACE. Quantitative PCR was performed using SYBR[®] Green chemistry on a MyiQTM Real-Time PCR Detection System (Bio-Rad, NSW, Australia). Each reaction (25 μ L) contained primers (200 nM each), 1 \times SensiMix^{Plus} SYBR & Fluorescein PCR master mix (Quantace) and 2 μ L cDNA. All samples were assayed for each gene in duplicate with no-template controls and a 5-step, 2-fold cDNA dilution series for PCR efficiency calculation on the same plate. The reaction was incubated at 95°C for 10 min to activate the heat-activated Taq DNA polymerase followed by 40 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 25 s. At the end of the 40 cycles a melt curve analysis was performed to test the specificity of reaction.

Relative expression

For the first experiment on gene expression (Chapter 9) mRNA expression levels were normalised using the stably expressed reference gene EF1A. Automated analysis of real-time quantitative PCR data was performed using REST (Relative Expression Software Tool) (Pfaffl, 2002) which employs a modified delta delta-Ct relative

quantification model with PCR efficiency correction and reference gene normalisation and incorporates a pair-wise fixed reallocation randomisation test for statistical significance ($P < 0.05$). In further experiments (Chapter 5 and 6) mRNA expression levels were normalized using the geometric mean of three stably expressed reference genes (eukaryotic elongation factor 1 alpha (EF1A), beta actin (β -actin), and RNA polymerase II (RPL2) as determined by the geNorm software (Vandesompele, 2002). Automated analysis of real-time quantitative PCR data was performed using qBase software (Hellemans, 2007) which employs a modified delta delta-Ct relative quantification model with PCR efficiency correction and multiple reference gene normalisation.

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Table 1.1

Selected proteolytic and housekeeping genes and their respective proteolytic pathway

Gene	Proteolytic pathway	Species
Cathepsin D (CatD)	Lysosomal	<i>S. salar</i> <i>L. calcarifer</i>
Cathepsin L (CatL)	Lysosomal	<i>S. salar</i> <i>L. calcarifer</i>
Polyubiquitin (PolyUb)	Ubiquitin-proteasome	<i>S. salar</i>
β proteasome subunit N3 (ProtN3)	Ubiquitin-proteasome	<i>S. salar</i> <i>L. calcarifer</i>
Calpain 1 (Capn1)	Calpain	<i>S. salar</i> <i>L. calcarifer</i>
Calpain 2 (Capn2)	Calpain	<i>S. salar</i> <i>L. calcarifer</i>
β actin	Housekeeping gene	<i>S. salar</i> <i>L. calcarifer</i>
Eukaryotic elongation factor 1 α (EF1a)	Housekeeping gene	<i>S. salar</i> <i>L. calcarifer</i>
β 2 microglobulin (B2M)	Housekeeping gene	<i>S. salar</i> <i>L. calcarifer</i>
RNA polymerase II (RpoI2)	Housekeeping gene	<i>S. salar</i>

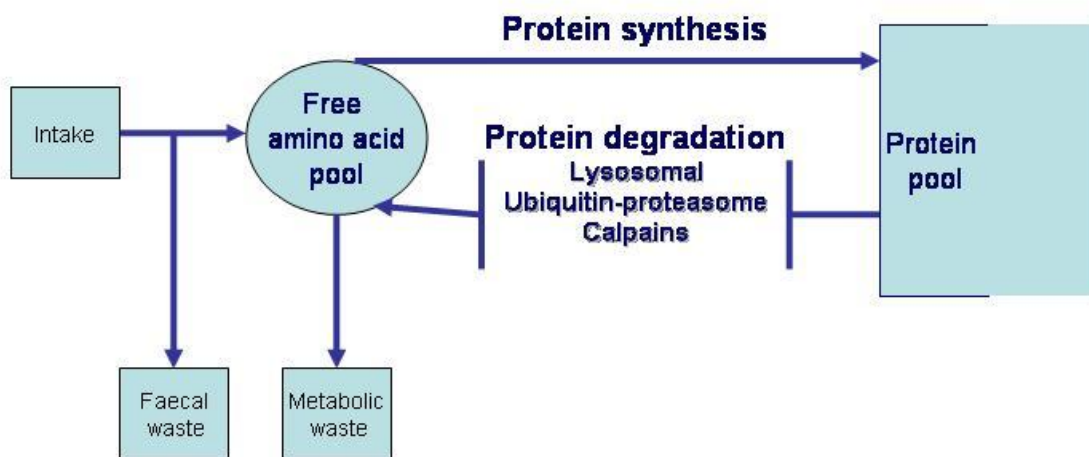
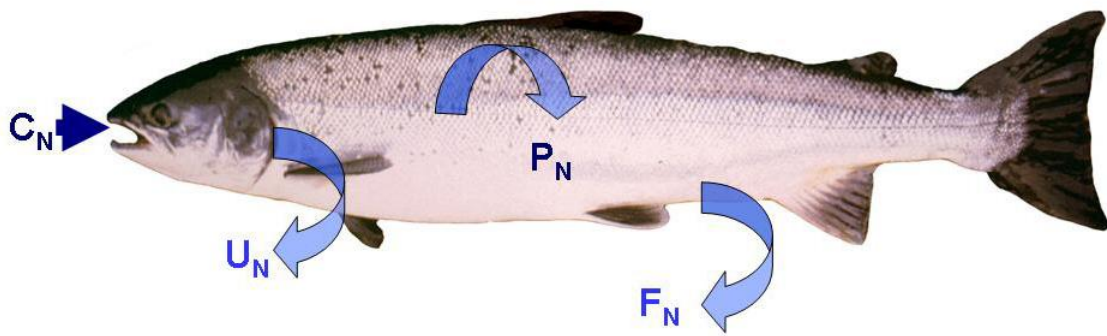


Fig. 1.1.
Protein-nitrogen flux and protein turnover.

Chapter 2

Optimum dietary protein to energy ratio for seawater Atlantic salmon, *Salmo salar* L., at an elevated temperature of 19°C**Abstract**

The optimum dietary digestible protein (DP) to digestible energy (DE) ratio for seawater Atlantic salmon (*Salmo salar* L.), initial wet weight $140.5 \text{ g} \pm 0.47 \text{ g}$, was determined at an elevated water temperature of 19°C. Four extruded diets were produced on a commercial extruder and vacuum coated with fish oil. Four diets were formulated to be either high (H) or low (L) in protein and energy: L:L (low protein and low energy); L:H (low protein and high energy); H:L (high protein and low energy) and H:H (high protein and high energy). Each diet was fed to satiation in triplicate, and to further groups at 60% and 30% of satiation. Two additional tanks were starved for 25 days to estimate maintenance requirements. Salmon fed to satiation consumed different weights of the diets but had similar digestible energy intake. Whole body dry matter and lipid content were significantly higher for H:L than for L:H whereas protein and ash were not affected by diet. Diet did not significantly influence the linear relationships between weight gain and nutrient intake so data were pooled. Maintenance requirements for wet weight were calculated as $4.58 \text{ g DP.kg}^{-0.7}.\text{d}^{-1}$ and $155.35 \text{ kJ DE.kg}^{-0.8}.\text{d}^{-1}$. Above maintenance, the efficiencies for protein and energy gain were 53% and 67%, respectively. These data predicted the optimum DP:DE for wet weight gain for seawater Atlantic salmon at 19°C was $19.77 \text{ g DP.MJ DE}^{-1}$. This information provided the optimum dietary formulation for use under commercial conditions.

2.1. Introduction

Atlantic salmon (*Salmo salar* L.) are farmed from temperate and polar regions and account for a significant proportion of global aquaculture production (Carter, 2007; Jobling et al., 2008). Climate change means that higher water temperatures are being experienced in many locations around the world, elevated summer temperatures are likely to increase the occurrence of adverse events on Atlantic salmon performance and hence its aquaculture. In some areas, such as Tasmania Australia, summer water temperatures can rise significantly above the optimum for growth of seawater salmon (Carter et al., 2005; Miller et al., 2006). Whilst higher temperatures in Tasmania have resulted in higher growth the benefits are easily lost through poorer performance during a warmer summer (Linton et al., 1998).

Fish are ectotherms and growth rate is strongly influenced by water temperature which, along with fish size, is the most important factor influencing fish growth when the food supply is not limiting (Brett, 1979; Elliott, 1979). The influence that temperature has on the dynamics between feed intake, metabolism and growth have been well described in conceptual terms (Brett, 1979; Elliott, 1979; Jobling, 1993,1994) and in detail for specific species (Elliott, 1991; Katersky and Carter, 2007). Metabolism, and therefore metabolic losses, increases exponentially with temperature where as feed intake initially increases with temperature but reaches a peak value from which it falls rapidly over a small further increase in temperature. This means that the optimum temperature for growth is predicted to be slightly below that of the maximum feed intake. Whilst a number of studies have investigated the effect of temperature on the growth performance of Atlantic salmon (Jensen et al., 1989; McCarthy and

Houlihan, 1996; Rungruangsak-Torrissen et al., 1998; Grisdale-Helland et al., 2002), prior to the current research, little research on the optimum diet composition at elevated temperature was available.

Factorial modelling, where nutrient requirements are expressed in terms of intake per unit of weight or weight gain, allow more accurate estimates of requirements to be made than when requirements are expressed as a proportion of a diet (Hauler and Carter, 2001; Lupatsch et al., 2001a). Factorial modelling takes account of allometric changes through the use of weight exponents for both energy and protein. A weight exponent of 0.8 for energy appears relatively consistent as judged by a large number of empirical studies (Brett and Groves, 1979; Hepher, 1988). Whilst there are fewer examples of a weight exponent for protein, a value of 0.7 appears generally applicable (Brett and Groves, 1979; Hepher, 1988; Lupatsch et al., 2001a; Lupatsch et al., 2001b). Furthermore, weight exponents are not greatly affected by water temperature. Consequently, exponents of 0.8 and 0.7 for energy and protein, respectively, are used in the present experiment for Atlantic salmon at 19°C. The effect of diet composition on the growth and nutrient utilisation efficiency of seawater Atlantic salmon at elevated water temperatures has not been fully explored. Determining the optimum DP:DE ratio required at elevated temperatures provides important information for feed formulation appropriate to locations around the world in which salmon are currently farmed. The aim of the present study was to determine the effect of DP:DE ratio on the growth and, protein and energy utilisation efficiency on Atlantic salmon grown at 19°C by feeding four diets of different protein and energy. The diets were fed at satiation and at restricted rations to determine the response of growth to gradations in protein and energy intake.

2.2. Materials and methods

2.2.1. Experimental diets

Four experimental diets were produced using a commercial extruder (Wenger X185 Optima) and vacuum-coated with fish oil (Skretting, Cambridge, Tasmania, Australia). The diets were formulated to be either high (H) or low (L) in protein and energy (Table 2.1): L:L low protein low energy; L:H low protein high energy; H:L high protein low energy; H:H high protein high energy. The protein was supplied from anchovy meal (Austral Group, Peru), soybean meal (Hunter Grain, NSW, Australia), corn gluten meal (Hunter Grain, NSW, Australia), feather meal (Camelleri Stockfeeds, NSW, Australia) and whole wheat (Cooperative Bulk Handling, WA, Australia). Lipid was supplied from anchovy oil (Sindicato Pesquero, Peru). A vitamin/mineral premix (commercial preparation according to NRC 1993 requirements for salmonids) was added. To measure digestibility an inert marker, ytterbium oxide (Yb_2O_3 ; 0.5%DM (dry matter) of the diet w/w), was mixed with half the fish oil, this was then used to coat the feed “kernel”. The remaining fish oil was then added on over this as a top coat to reduce the loss of ytterbium to the water. Feeds were stored at 1 to 2°C for the duration of the experiment.

2.2.2. Growth experiment

The experiment was conducted at the School of Aquaculture, University of Tasmania. Atlantic salmon (*Salmo salar* L.) smolt were obtained from Wayatinah

Salmon Hatchery (SALTAS, Tasmania, Australia) and held in a 4000-l freshwater tank. They were then evenly divided between twenty-five 300-l tanks in a partial recirculation system (Carter and Hauler, 2000) and acclimated to saltwater (30 ppt) over 6 days in increments of 5 ppt. The temperature was then raised from 12°C to 19°C over 12 days. During acclimation fish were fed the H:L diet. Water was treated through physical and bio-filters with partial water changes as required to ensure water quality parameters (DO, pH, ammonia, nitrite and nitrate) stayed within the limits recommended for seawater Atlantic salmon (Tarazona and Munoz, 1995).

At the start of the experiment, fish were anaesthetised (50 mg l⁻¹, benzocaine) and weight and fork length measured. Fish weighing less than 90 g or more than 200 g, or with external damage were not used. Selected fish were randomly allocated to one of twenty-two 300-l tanks to ensure there were no significant differences between group mean weights (140.5 ± 0.5 g). One fish from each tank was killed and 9 of these were randomly taken for an assessment of initial chemical composition (see below). The tanks were randomly allocated to a diet and ration. Each of the four diets was allocated 5 tanks, 3 fed to satiation, 1 to 60% of satiation and 1 to 30% of satiation. Two more tanks were starved for 24 days to determine maintenance requirements from the change in whole body chemical composition under these conditions.

The fish were fed at 0900 and 1600 every day and feed intake determined using collectors that were placed over each tank's water outlet. The extruded pellets remained intact prior to collection and uneaten feed was estimated from the number of leftover pellets using the average weight of a pellet for each feed (Helland et al., 1996). Feed supplied to the satiation tanks was always in excess. The 60% and 30% rations were calculated from the previous day's average measured feed intake at satiation for that diet. The fish were re-weighed every 21 days and the experiment continued for 104 days, by which time the satiation-fed fish had more than tripled in weight. Prior to the end of the experiment the fish were not fed for a day. All fish were anaesthetised and weight and fork length measured. Fish were euthanised by an overdose of anaesthetic (benzocaine) and taken for analysis of whole body chemical composition and tissue samples (see below).

All calculations were based on tank means from the satiation treatments. Specific growth rate (SGR) was calculated as $SGR (\% d^{-1}) = 100((\ln (W_2/W_1)) / t)$, where W_1 and W_2 are the weights (g) at two times and t the number of days. Feed, protein and energy efficiency ratios (FER, PER, EER) were calculated at the end of the experiment as $ER (g.g^{-1} \text{ or } g.kJ^{-1}) = ((W_f - W_i) / N_{intake})$ where W_f and W_i are the final and initial wet weight in grams and N_{intake} is the intake of nutrient: feed (g), protein (g) or energy (kJ) consumed. Digestible protein and digestible energy efficiency ratios (DPER and DEER) were calculated using the above formula, with N_{intake} being digestible nutrient value. The efficiency with which consumed protein and energy were retained was calculated as productive protein value (PPV) and productive energy value (PEV) so that $PV (\%) = 100((N_f - N_i) / N_{intake})$ where N_f and N_i are the final and initial amounts of the nutrients in the fish and N_{intake} is the amount of the nutrient consumed by the fish. The digestible PVs (DPPV and DPEV) were also calculated using the above formula and digestible nutrient values.

2.2.3. Apparent digestibility

Apparent digestibility (AD; %) was measured at the end of the experiment using all tanks in the growth trial. The fish were fed the ytterbium-coated feeds for 8 days and for the last 6 days faecal samples were collected by settlement using collectors attached to the tanks between 1700 and 0900h (Carter and Hauler, 2000; Ward et al., 2005). The samples were freeze-dried, ground with a mortar and pestle and fish scales removed. Feed samples were also freeze-dried and ground. These samples were used in the analysis of nutrients and ytterbium marker (see below). Apparent digestibility was calculated as $AD (\%) = 100 - (100 (\%I_{\text{diet}} / \%I_{\text{faeces}}) \times (\%N_{\text{faeces}} / \%N_{\text{diet}}))$ (Maynard and Loosli, 1969) where I is the inert marker and N the nutrient in the diet and faeces. The digestible protein and energy values were applied to the crude protein and gross energy data to determine the digestible protein (DP) and digestible energy (DE) content of the 4 diets.

2.2.4. Chemical analysis

Standard methods were used to determine dry matter (freeze dried to constant weight); nitrogen (Kjeldahl using a copper catalyst); total lipid (Bligh and Dyer, 1959); energy (bomb calorimeter: Gallenkamp Autobomb, calibrated with benzoic acid); ash (AOAC, 1995). Ytterbium concentrations in the feed and faeces were determined using Flame Atomic Absorption Spectrometry (Varian AAS) (Ward et al., 2005).

2.2.5. Statistical analysis

Data are reported mean \pm SEM (standard error of the means). Comparison between means from satiation treatments (n=3) was by one-way ANOVA, with post-hoc analysis by Tukey. The slopes of the regression lines were compared by ANCOVA; where there was no significant difference between treatments the data were pooled. Significance was accepted at probabilities of < 0.05 . Analyses were carried out with SPSS for Windows.

2.3. Results

2.3.1. Digestible nutrient intake

The apparent digestibility of protein, lipid and energy were more than 89%, 94% and 88%, respectively (Table 2.2). Protein apparent digestibility for the L:L diet was significantly lower than that for H:L, with L:H and H:H not significantly different from either. AD energy for L:L was significantly lower than for the other 3 diets. There was no significant difference in lipid digestibility between diets.

At satiation the overall feed intake for L:L was significantly greater than for L:H, with H:L and H:H not significantly different from either (Table 2.3) and there was a significant inverse relationship between dietary digestible energy content and feed intake, so that fish on lower energy diets consumed more in terms of %BW (Fig. 2.1a). Consequently total DE intake was not significantly different between treatments (Fig 2.1a: $F = 2.21$, $P = 0.17$, $n = 3$). In contrast, there was no relationship between dietary digestible protein content and feed intake in terms of %BW (Fig. 2.1b).

2.3.2. Growth and body composition

Mortalities were 10% or less with no significant difference between treatments. Fish grew consistently over the course of the experiment, with wet weight increasing by at least 3-fold. There was no significant difference in final wet weight, wet weight gain or specific growth rate (SGR) among the diets (Table 2.3). Dry matter and total lipid of the fish were significantly higher for L:L than for H:L but there was no significant differences among crude protein, ash or gross energy (Table 2.4). Whole body protein and non-protein energy were approximately equal and there were no significant differences among diets. Fish starved for 24 days decreased wet weight by $10.42 \text{ g.kg}^{-0.8} \cdot \text{d}^{-1}$, protein by $0.817 \text{ g.kg}^{-0.7} \cdot \text{d}^{-1}$ and energy by $124.1 \text{ kJ.kg}^{-0.8} \cdot \text{d}^{-1}$.

Efficiency expressed as either feed efficiency ratio (FER) or protein efficiency ratio (PER) were not significantly different between the diets whereas the energy efficiency ratio (EER) was significantly higher for H:H than for L:H (Table 2.5). The productive value for crude protein (PPV) and gross energy (PEV) were not significantly different between the diets. However, digestible energy was retained more efficiently on H:H than on L:H.

2.3.3. Modelling feed intake and growth

There was no significant difference between the diets for protein gain ($F=2.009$, $P=0.191$, $n=3$) or energy gain ($F=3.801$, $P=0.058$, $n=3$). Data from satiation and restricted treatments were combined to make an analysis of the relationships between nutrient intake and growth. There were no significant differences (ANCOVA) between the slopes of the regression lines so the data from the different diets were pooled and a regression equation recalculated. There were significant linear relationships between weight gain and feed intake (Fig 2.2), digestible protein intake (Fig 2.3) and digestible energy intake (Fig 2.4). These relationships predicted wet weight maintenance requirements at 19°C for protein and energy of $4.56 \text{ g DP.kg}^{-0.7} \cdot \text{d}^{-1}$ and $155.35 \text{ kJ DE.kg}^{-0.8} \cdot \text{d}^{-1}$, respectively.

The relationship between digestible nutrient intake and nutrient retention was analysed for protein and energy. There were significant relationships between digestible protein intake and protein retention (Fig 2.5) and between digestible energy intake and retention (Fig 2.6). Accordingly maintenance requirements for whole body protein and energy gain were $2.19 \text{ g DP.kg}^{-0.7} \cdot \text{d}^{-1}$ and $184.77 \text{ kJ DE.kg}^{-0.8} \cdot \text{d}^{-1}$, respectively. The slope of the regression represents a cost of production and equated to 1.87 and 1.49 for one unit of protein and energy, respectively. The reciprocal value ($1/1.87$ and $1/1.49$) described the efficiency of utilisation for growth above maintenance, so that for digestible protein efficiency was 53% and 67% for digestible energy.

There was no significant difference in growth rate of the satiation-fed fish and the average growth rate was used to determine the protein and energy requirements for a fish growing at maximal potential. The maximum growth rate was $36.76 \text{ g.kg}^{-0.8} \cdot \text{d}^{-1}$, protein gain was $11.59 \text{ g.kg}^{-0.7} \cdot \text{d}^{-1}$ and energy gain was $346.69 \text{ kJ.kg}^{-0.8} \cdot \text{d}^{-1}$. The feed intake (DM) required to support this growth rate for these fish was $34.27 \text{ g.kg}^{-0.8} \cdot \text{d}^{-1}$. The digestible protein requirement for maximum weight gain was $25.05 \text{ g.kg}^{-0.7} \cdot \text{d}^{-1}$, and for digestible energy, $693.40 \text{ kJ.kg}^{-0.8} \cdot \text{d}^{-1}$. This equated to a DP:DE of 19.77 g

DP.MJ DE⁻¹ for 464 g Atlantic salmon at 19°C. The protein and energy requirements for protein and energy gain were similar to those for weight gain, being 13.73 g.kg^{-0.7}.d⁻¹ and 703.72 kJ.kg^{-0.8}.d⁻¹, respectively.

2.4. Discussion

Nutrient requirement determinations have usually been carried out under optimum conditions under which fish exhibit high growth rates (Carter et al., 2005). In the present experiment the optimum dietary digestible protein to digestible energy ratio and parameters related to protein and energy utilisation were determined for seawater salmon at an elevated temperature of 19°C. This temperature was described as being elevated because it is above the optimum for Atlantic salmon (Forseth et al., 2001). In Tasmania, and increasingly elsewhere in the world, farmed Atlantic salmon of a similar size experience water temperatures of 19°C (Carter et al., 2005; Miller et al., 2006). At this temperature they are fed commercially and appear to have the capacity to grow relatively well, it is, therefore, important to determine the optimum diet in order to achieve efficient growth. Despite the size of Atlantic salmon aquaculture there are relatively few published data on the optimum temperature for growth of Atlantic salmon, optimum temperatures of between 12 and 15°C for freshwater and seawater stages are given in relation to farming (Isaksson, 1991). Wild Atlantic salmon parr collected from a cold glacier fed river in western Norway had an optimum temperature of 18-19°C which was considered slightly higher than wild parr collected from UK streams (Forseth et al., 2001). Optimum temperature decreases with size and the temperature used in the present experiment was likely to be above the optimum for these salmon, in commercial terms it was above the optimum range which is less than 18°C and considered to be around 14-16°C.

It is important to note that the four diets were determined to have most commercial significance, they were formulated by the commercial partner on the production line used for current commercial salmon feeds. In relation to the L:L diet, the high protein diets (H:L and H:H) had approximately 10% more crude protein and 10% more digestible protein. In relation to the L:L diet, the low protein: high energy diet (L:H) had 9% more gross energy and 10% more digestible energy. The difference in energy was less distinct between the two high protein diets, the high energy diet (H:H) had 3% more energy and the same digestible energy as the low energy diet (H:L). An important advantage of using a factorial analysis aimed to determine the protein and energy requirements in relation to growth meant that differences in diet formulations, whatever their magnitude, can be modelled and contribute to predicting the amount of nutrient required for a unit of growth.

2.4.1. Feed intake

A number of abiotic and biotic factors influence the feed intake of fish, the most important in an aquaculture context are temperature, fish weight and diet composition (Brett, 1979; Brett and Groves, 1979). Feed intake increases with temperature to a maximum that is often slightly above that of the optimum for growth (Jobling, 1994; Katersky and Carter, 2007), however this may not always be the case and wild Atlantic salmon parr had maximum feed intake and growth at similar temperatures (Forseth et al., 2001). In the latter example maximum feed intake occurred at between 19.5 and 19.8°C. Maximum feed intake scales with weight with an exponent of between 0.6 and

0.8, an exponent of 0.75 is considered generally applicable and consequently the relationship between weight specific feed intake and weight scales with an exponent of -0.25 (Jobling, 1994).

In the current experiment, and across the range of DP and DE, the data very clearly showed that Atlantic salmon regulated feed intake in relation to dietary digestible energy. At satiation there was no difference in the intake of DE between the four diets whereas feed intake on a DM basis decreased with increasing dietary DE. The data were less clear in relation to intake of DP, neither DP nor DM intake were correlated with dietary DP. The generally accepted view is that energy intake is the key determinant of feed intake, fish and other animals are thought to eat to meet their energy requirement. However, it also clear that nutritional factors such as nutrient deficiency, dietary imbalances and dietary protein will also influence intake (Forbes, 1999; Simpson and Raubenheimer, 2001).

2.4.2. Digestible nutrient intake

Most studies investigating apparent digestibility (AD) in Atlantic salmon have been conducted at lower temperatures than the current experiment. However, AD values for crude protein, total lipid and gross energy from the present experiment were typical of similar studies on Atlantic salmon and other salmonids using similar ingredients (Anderson et al., 1995; Sugiura et al., 1998; Carter and Hauler, 2000; Sajjadi and Carter, 2004). Temperature probably has the greatest influence on AD lipid due to solidification of saturated fats at low temperatures. Temperature may also influence AD carbohydrate due to changes in characteristics of starch at different temperatures (Guillaume et al., 1999). Wide variation amongst experimental designs prevent generalisation about the effect of temperature on AD. At a biological level, increases in physiological processes such as digestive enzyme activity and gastric evacuation due to increased temperature are likely to be balanced by the increased volume of feed consumed so that digestibility values are not influenced greatly by temperature.

In the current experiment dietary crude protein content significantly affected AD crude protein, at low crude protein (41.5%; L:L diet) protein AD was significantly lower than at the highest crude protein (46.2%; H:L diet). However, the numerical difference was in the order of 1-2% and the biological significance was not clear since there were no differences in growth amongst the diets. AD crude protein was not correlated with dietary protein content or with protein intake for a range of protein ingredients fed to rainbow trout and Coho salmon (Sugiura et al., 1998). In the current experiment total lipid was well digested and there were no significant differences between the diets. The diets contained about 13.5% plant protein sources and 15% wheat that contained a significant indigestible carbohydrate fraction so that AD energy was lower than AD crude protein and lipid. There were differences in AD energy between diets and these reflected the differences in AD crude protein.

2.4.3. Body composition

Whole body protein remains relatively constant and is not strongly influenced by abiotic or biotic factors (Shearer, 1994; Shearer et al., 1994). More specifically, temperature did not affect protein content of different salmonids (Elliott, 1979; Shearer, 1994). "Baltic" Atlantic salmon (*Salmo salar* L.) had the same protein content

from 11 to 23°C (Koskela et al., 1997) and the results from the present study are similar to Atlantic salmon grown at lower temperatures (Sveier et al., 2000; Nordgarden et al., 2002). Reasons that explain why whole body protein remains relatively constant is that lipid and water contents are inversely related and fish lay down lipid as an energy store (Love, 1957; Shearer, 1994). Thus, increasing the whole body lipid replaces water and does not affect protein content. Whole body lipid content is more variable and strongly influenced by the nutritional status of the fish, which is largely determined by nutrient intake (Einen and Roem, 1997; Azevedo et al., 2002; Azevedo et al., 2004). In the present experiment the lower DP:DE diets resulted in fish with higher lipid content. Whole body lipid content increased in Atlantic salmon, rainbow trout and chinook salmon as dietary DP:DE decreased (Azevedo et al., 2002).

Temperature affects energy status and therefore lipid content, the principle mechanism is through its influence on metabolic rate and overall energy balance (Brett and Groves, 1979). An important feature of the current experiment was that the fish were fed to satiation and differences between diets controlled appetite and feed intake. This meant that the data on whole body composition reflect the composition set by the fish.

2.4.4. Daily protein and energy requirements

The diets used represented a range of DP:DE ratios found in current commercial feed preparations. Comparison of the mean response showed the diets resulted in similar growth, which suggested that DP:DE ratios from 18 to 21 g.MJ⁻¹ were sufficient for good growth performance at 19°C. Similarly, the various measures of whole body composition and of efficiency for weight, protein and energy gain were similar between diets. As highlighted above, the major difference between diets was their effect on feed intake. Growth and body composition were then determined by nutrient intake, the combination of feed intake and diet composition.

The relationships between wet weight gain and feed, protein and energy intake were used to estimate the feed, protein and energy requirements for wet weight gain. Similarly, the relationships between nutrient retention and digestible nutrient intake allowed calculation of the maintenance requirements for protein and energy and their utilisation efficiency above maintenance.

Protein and energy requirements for maintenance of wet weight were 4.58 g.kg^{-0.7}.d⁻¹ and 179.13 kJ.kg^{-0.8}.d⁻¹, respectively. The efficiency of utilisation of digestible protein for growth above the maintenance requirement was 0.53 (53%) and similar to several other species: 0.52 for European sea bass, *Dicentrarchus labrax* (Lupatsch et al., 2001a); 0.56 for common carp, *Cyprinus carpio* (Schwarz and Kirchgessner, 1995); 0.54 for rainbow trout (Rodehutscord and Pfeffer, 1999). These experiments were carried out at different temperatures which suggested temperature does not significantly affect utilisation efficiency of protein in fish. The efficiency of utilisation of digestible energy for growth above the maintenance requirement was 0.67 (67%) and also similar to the value found for other fish species: 0.68 for European sea bass (Lupatsch et al., 2001a); 0.61 (Azevedo et al., 1998) and 0.68 (Rodehutscord and Pfeffer, 1999) for rainbow trout. Energy utilisation was independent of temperature, feeding level and fish size (Azevedo et al., 1998) and the current experiment supports this conclusion.

Maintenance requirements were also calculated for wet body protein and energy content. According to the regression equations, protein and energy gain were zero (maintenance) at DPI of $2.19 \text{ g DP.kg}^{-0.7} \cdot \text{d}^{-1}$ and DEI of $184.77 \text{ kJDE.kg}^{-0.8} \cdot \text{d}^{-1}$, respectively. Therefore the protein requirement to maintain wet weight ($4.58 \text{ g DP.kg}^{-0.7} \cdot \text{d}^{-1}$) was more than that needed to maintain protein content. Whereas the energy requirement to maintain wet weight ($179.13 \text{ kJ DE.kg}^{-0.8} \cdot \text{d}^{-1}$) was less than required to maintain energy content.

Maintenance requirements determined above from nutrient intake-gain relationships were greater than those determined using nutrient losses from unfed fish: $0.82 \text{ g DP.kg}^{-0.7} \cdot \text{d}^{-1}$ and $124.10.8 \text{ kJDE.kg}^{-0.8} \cdot \text{d}^{-1}$ for protein and energy, respectively. This is because starved fish utilise physiological mechanisms to minimise nutrient expenditure in an effort to conserve body weight in adverse conditions (see Chapter 4).

As discussed above there was no significant difference amongst diets in growth rate of satiation-fed fish, thus the average growth rate across all the diets was used to determine the protein and energy requirements for a 464 g Atlantic salmon growing at its maximum potential. The maximum growth rate was $36.76 \text{ g.kg}^{-0.8} \cdot \text{d}^{-1}$, protein gain was $11.59 \text{ g.kg}^{-0.7} \cdot \text{d}^{-1}$ and energy gain was $346.69 \text{ kJ.kg}^{-0.8} \cdot \text{d}^{-1}$. The feed intake required to support this growth was $34.27 \text{ g DM.kg}^{-0.8} \cdot \text{d}^{-1}$. For maximum wet weight gain the digestible protein required was $25.05 \text{ g DP.kg}^{-0.7} \cdot \text{d}^{-1}$ and the digestible energy was $693.40 \text{ kJDE.kg}^{-0.8} \cdot \text{d}^{-1}$. This result equates to a DP:DE of $19.77 \text{ g DP.MJ DE}^{-1}$ for 464g Atlantic salmon at 19°C. The protein and energy requirements for protein and energy gain were similar to those for wet weight gain, being $13.73 \text{ g DP.kg}^{-0.7} \cdot \text{d}^{-1}$ and $703.72 \text{ kJ DE.kg}^{-0.8} \cdot \text{d}^{-1}$, respectively.

The dietary effects of varied levels of protein and energy on Atlantic salmon growth and nutrient utilisation have traditionally been investigated on fish kept in water temperatures within optimal growth ranges. The present study has determined the effects at a temperature above the growth optimum for Atlantic salmon. This experiment determined the digestible requirement for protein and energy at 19°C, taking into account requirements for maintenance and growth. The optimal DP:DE for Atlantic salmon of the size reached by the end of the experiment was determined to be $19.77 \text{ g DP.MJ DE}^{-1}$.

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Table 2.1
Ingredient and chemical composition of experimental feeds

	Diet			
	L:L	L:H	H:L	H:H
Ingredient composition (g kg ⁻¹)				
Fish meal	427	427	534	480
Corn gluten	53	53	67	60
Soybean meal	82	82	102	92
Feather meal	44	44	56	50
Wheat	150	149	111	130
Vit/Min Premix	3	3	3	3
Fish oil	141	242	127	185
Bentonite	100	0	0	0
Chemical composition (g kg ⁻¹ DM)				
Dry matter (g kg ⁻¹)	93.89	92.72	92.39	93.28
Crude protein (g kg ⁻¹)	415.26	419.16	462.62	454.40
Total lipid (g kg ⁻¹)	288.99	347.53	314.18	298.24
Total ash (g kg ⁻¹)	138.79	80.86	92.58	85.24
Gross energy (MJ kg ⁻¹ DM)	21.81	23.74	22.72	23.29
Digestible protein (g.kg ⁻¹ DM)	379.42	382.80	425.38	411.03
Digestible energy (MJ.kg ⁻¹ DM)	19.45	21.40	20.36	20.60
DP:DE (g.MJ ⁻¹ DE)	19.50	17.89	20.90	19.96

Diets: L:L (low protein:low energy), L:H (low protein:high energy), H:L (high protein:low energy) and H:H (high protein:high energy).

Table 2.2

Apparent digestibility (AD%) for Atlantic salmon at 19°C of crude protein (N), lipid (L) and energy (kJ) from diets with different digestible protein digestible protein

Parameter		Diet				F	P
		L:L	L:H	H:L	H:H		
AD _N	(%)	89.62 ^a 0.42	90.88 ^{ab} 0.24	91.23 ^b 0.20	90.81 ^{ab} 0.03	5.33	0.026
AD _L	(%)	94.00 0.45	94.62 0.64	94.07 0.69	94.76 0.10	0.48	0.706
AD _{kJ}	(%)	88.18 ^a 0.31	88.90 ^b 0.45	89.58 ^b 0.20	90.02 ^b 0.19	6.376	0.016

Mean \pm SEM (n =3). Different letters denote significant (< 0.05) differences across rows. Diets: L:L (low protein:low energy), L:H (low protein:high energy), H:L (high protein:low energy) and H:H (high protein:high energy).

Table 2.3

Growth performance of Atlantic salmon at 19°C fed to satiation on diets with different DP:DE

Parameter	Diet				F	P
	L:L	L:H	H:L	H:H		
Wet weight initial (g)	139.64 (0.89)	140.65 (1.29)	141.29 (3.12)	140.56 (0.28)	0.15	0.926
Wet weight final (g)	510.16 (23.52)	417.90 (30.55)	449.13 (26.51)	477.47 (20.83)	2.37	0.147
Wet weight gain (g)	370.52 (22.76)	277.25 (31.84)	307.837 (28.38)	336.89 (21.01)	2.29	0.155
SGR (%.d ⁻¹)	1.24 (0.04)	1.04 (0.08)	1.12 (0.07)	1.17 (0.04)	2.00	0.190
Feed intake (g)	357.92 ^a (16.71)	259.64 ^b (11.86)	299.4 ^{ab} (28.52)	308.43 ^{ab} (11.89)	4.75	0.035

Mean \pm SEM (n =3). No significant (< 0.05) differences across rows. Diets: L:L (low protein:low energy), L:H (low protein:high energy), H:L (high protein:low energy) and H:H (high protein:high energy).

Table 2.4
Whole body chemical composition (% wet weight) of Atlantic salmon at 19°C fed diets with different DP:DE

Parameter			Diet				F	P
	Initial	Starved	L:L	L:H	H:L	H:H		
Dry matter (%)	31.96 (0.19)	30.88 (0.30)	35.32 ^a (0.30)	34.77 ^{ab} (0.29)	33.52 ^b (0.31)	34.62 ^{ab} (0.32)	6.08	0.018
Crude protein (%)	16.30 (0.03)	17.30 (0.16)	17.60 (0.21)	17.26 (0.31)	17.51 (0.39)	17.75 (0.18)	0.52	0.680
Total lipid (%)	13.30 (0.16)	10.85 (0.01)	15.66 ^a (0.55)	15.56 ^{ab} (0.47)	13.37 ^b (0.44)	14.86 ^{ab} (0.24)	5.77	0.021
Ash (%)	2.33 (0.05)	2.46 (0.11)	2.02 (0.04)	2.14 (0.11)	2.12 (0.09)	2.18 (0.06)	0.73	0.565
Gross energy (kJ.g ⁻¹)	7.96 (0.22)	7.18 (0.15)	9.10 (0.24)	8.55 (0.20)	7.93 (0.17)	8.03 (0.14)	2.42	0.142
Protein energy (kJ.g ⁻¹)	3.85 (0.01)	4.09 (0.04)	4.16 (0.05)	4.08 (0.07)	4.14 (0.09)	4.20 (0.04)	0.52	0.681
Non-protein energy (kJ.g ⁻¹)	4.12 (0.21)	3.09 (0.12)	4.94 (0.20)	4.84 (0.25)	4.28 (0.15)	4.70 (0.18)	5.76	0.166

Mean ± SEM (n =3). Different letters denote significant (< 0.05) differences across rows.

Diets: L:L (low protein:low energy), L:H (low protein:high energy), H:L (high protein:low energy) and H:H (high protein:high energy).

Table 2.5
Nutrient efficiencies of Atlantic salmon at 19°C fed different DP:DE diets

Parameter	Diet				F	P
	L:L	L:H	H:L	H:H		
FER (g.g ⁻¹)	1.03 (0.02)	1.06 (0.08)	1.03 (0.003)	1.09 (0.05)	0.36	0.784
PER (g.g ⁻¹)	2.50 (0.04)	2.53 (0.19)	2.21 (0.01)	2.40 (0.12)	1.57	0.271
EER (g.MJ ⁻¹)	47.46 ^{ab} (0.80)	38.61 ^a (4.44)	45.29 ^{ab} (0.14)	54.55 ^b (1.10)	7.99	0.009
PPV (%)	45.17 (0.45)	44.92 (3.66)	39.97 (1.14)	44.07 (2.59)	1.09	0.408
PEV (%)	45.95 (0.89)	44.30 (1.11)	39.84 (0.84)	43.02 (3.74)	1.60	0.263
DPER (g.g ⁻¹)	2.73 (0.05)	2.77 (0.21)	2.41 (0.01)	2.41 (0.13)	1.71	0.241
DEER (g.MJ ⁻¹)	53.21 ^{ab} (0.90)	43.66 ^a (5.02)	50.56 ^{ab} (0.15)	60.52 ^b (1.22)	7.05	0.012

Mean ± SEM (n =3). Different letters denote significant (< 0.05) differences (n=3).

Diets: L:L (low protein:low energy), L:H (low protein:high energy), H:L (high protein:low energy) and H:H (high protein:high energy) at 19°C.

FER (feed efficiency ratio), PER (protein efficiency ratio), EER (energy efficiency ratio) using equation: $ER (g.g^{-1} \text{ or } g.kJ^{-1}) = (W_f - W_i) / N_{intake}$

PPV (productive protein value) and PEV (productive energy value) using equation:

$PV (\%) = 100 \times ((N_f - N_i) / N_{intake})$

DPER (digestible protein efficiency ratio) and DEER (digestible energy efficiency ratio) using equation: $ER (g.g^{-1} \text{ or } g.kJ^{-1}) = (W_f - W_i) / \text{digestible } N_{intake}$

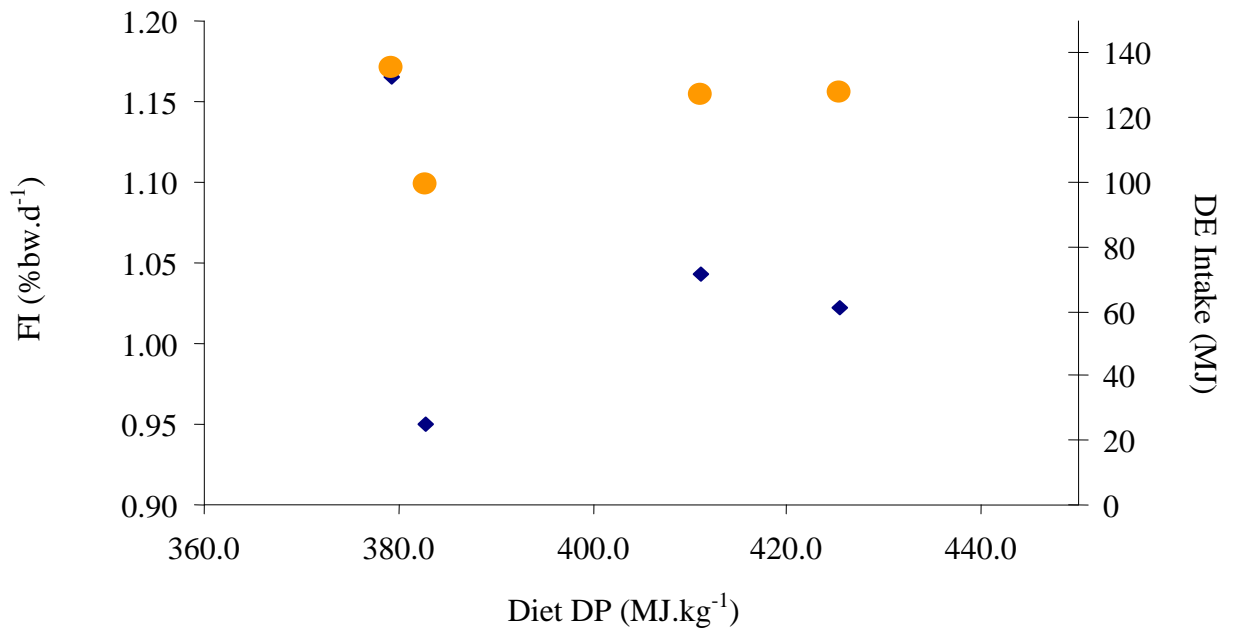
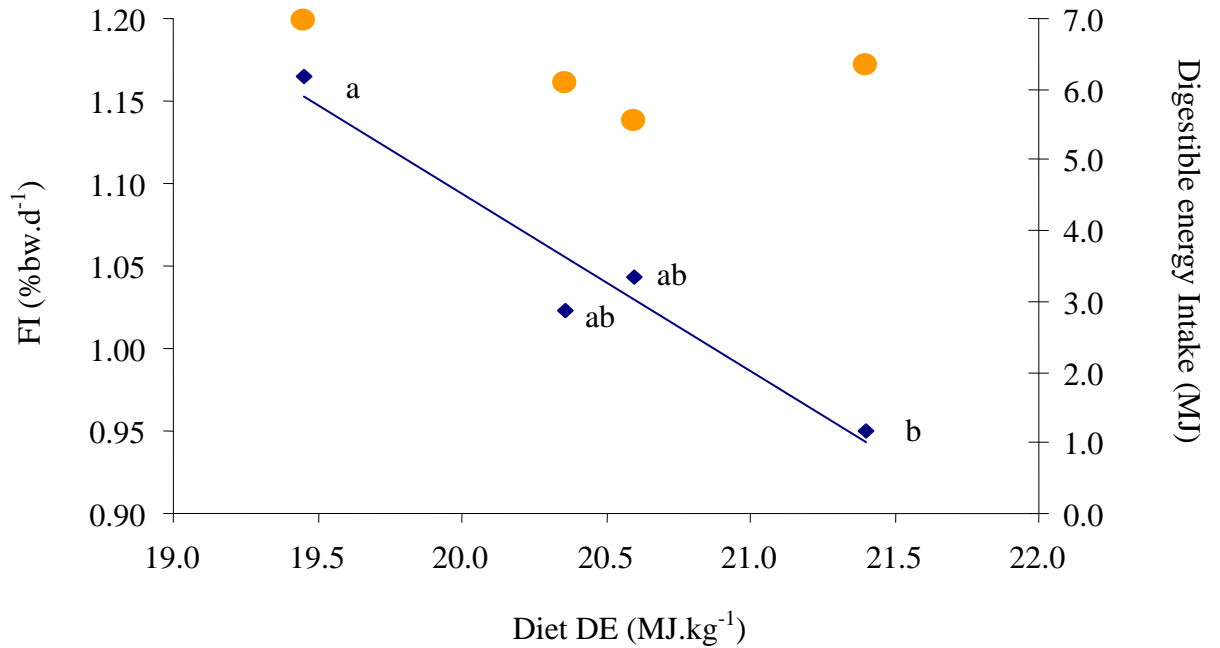


Fig. 2.1. Feed intake (FI %bw.d⁻¹ ◆) and total nutrient intake (●) of Atlantic salmon at 19°C in relation to (a) dietary digestible energy and (b) dietary digestible protein content. Different letters denote significant (< 0.05) differences. $FI (\%bw.d^{-1}) = -0.11 (\pm 0.02) DE + 3.25 (\pm 0.39) (R^2 = 0.94, F = 31.41, P = 0.03, n = 4)$.

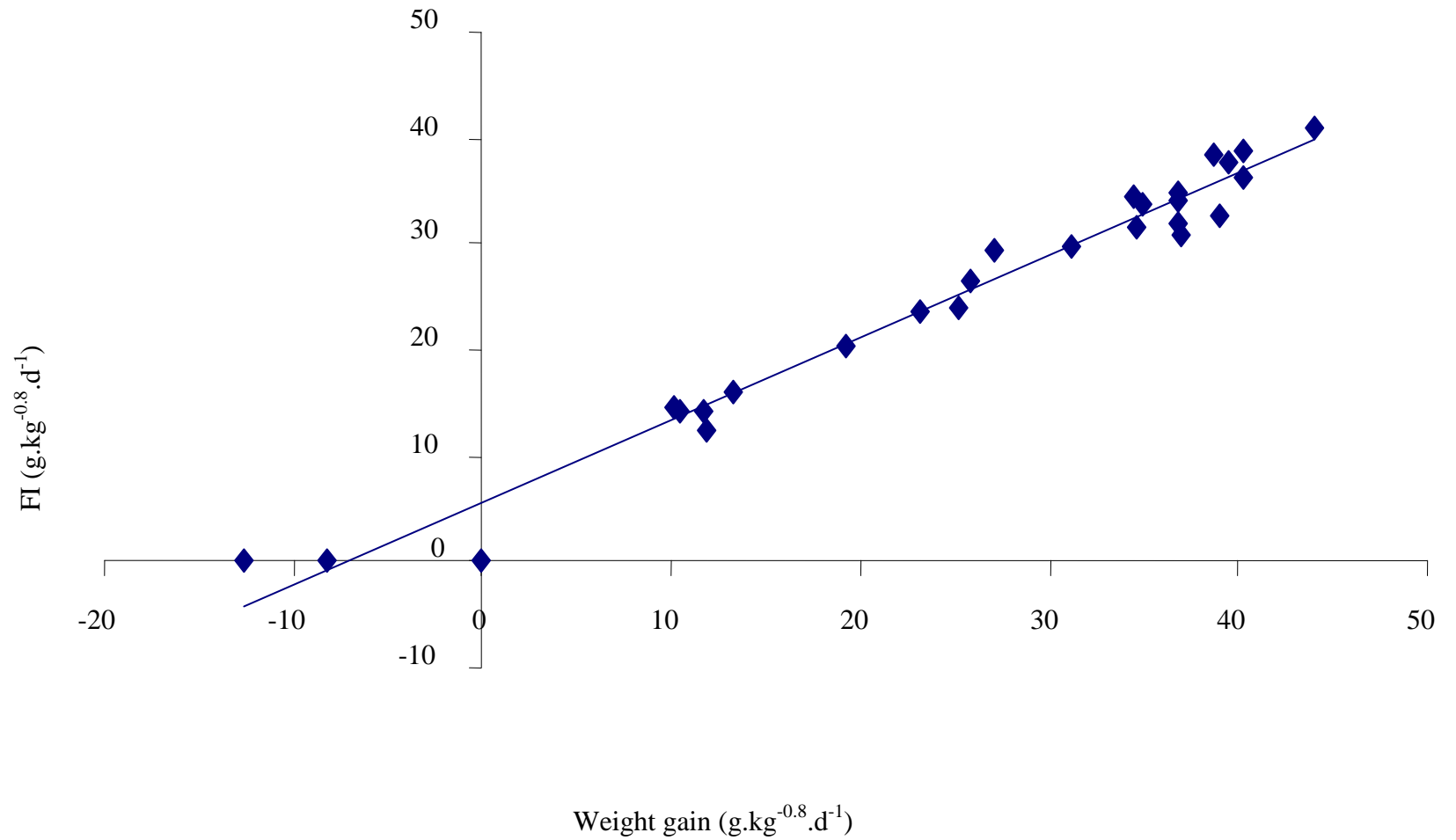


Fig. 2.2. The relationship between weight gain and feed intake of Atlantic salmon at 19°C fed diets of different DP:DE (Feed intake (g.g^{-0.8}.d⁻¹) = 0.75(0.02). Weight gain (g.g^{-0.8}.d⁻¹) + 6.88(0.72) (R² = 0.98, F = 934.181, P = 0.000, n = 22)).

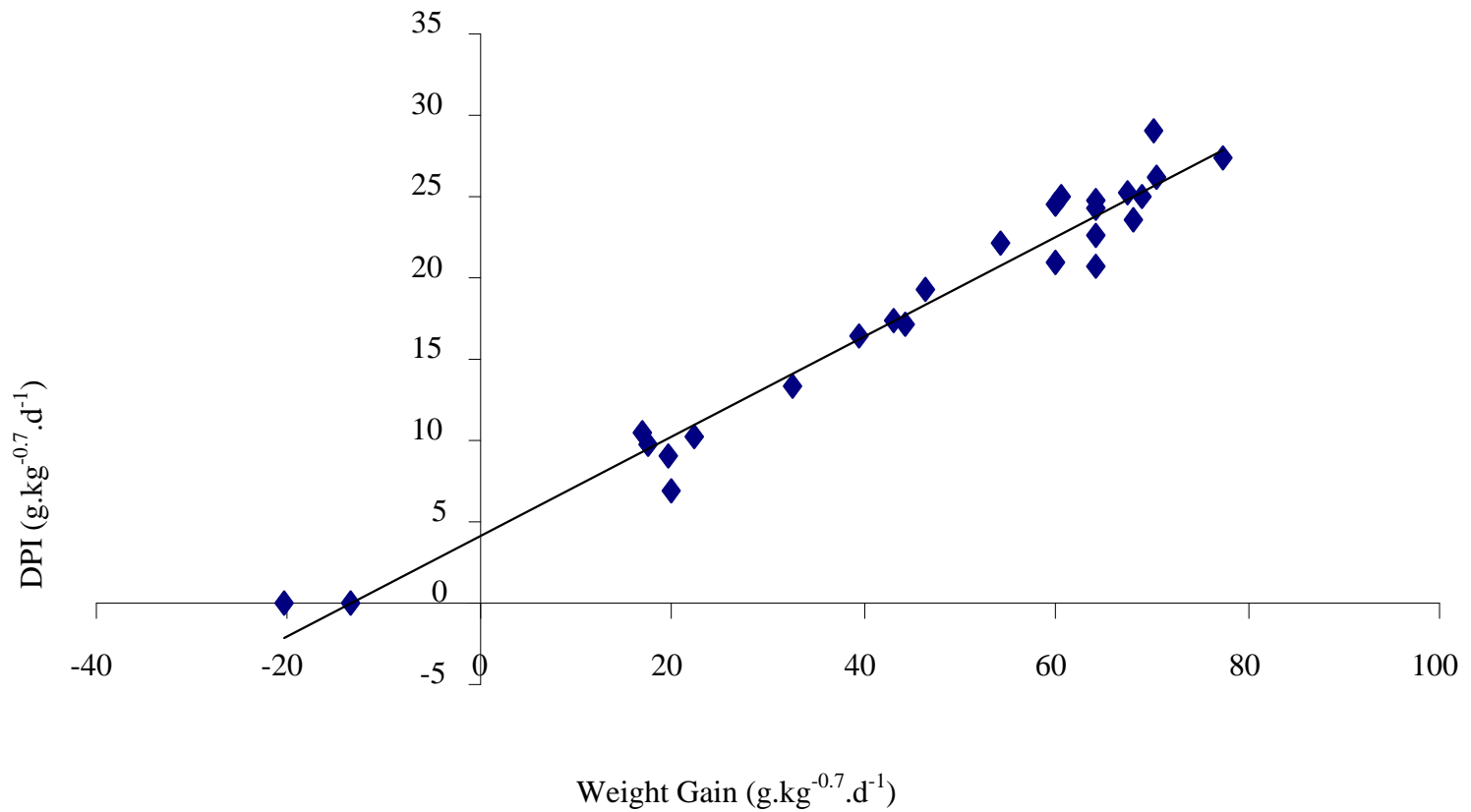


Fig. 2.3. The relationship between daily digestible protein intake (DPI) and weight gain of Atlantic salmon at 19°C fed diets of different DP:DE (DPI (g.kg^{-0.7}.d⁻¹) = 0.30 (±0.01). Weight gain (g.kg^{-0.7}.d⁻¹) + 4.58 (±0.63) (R² = 0.97, F = 611.5, P = 0.000, n = 22)).

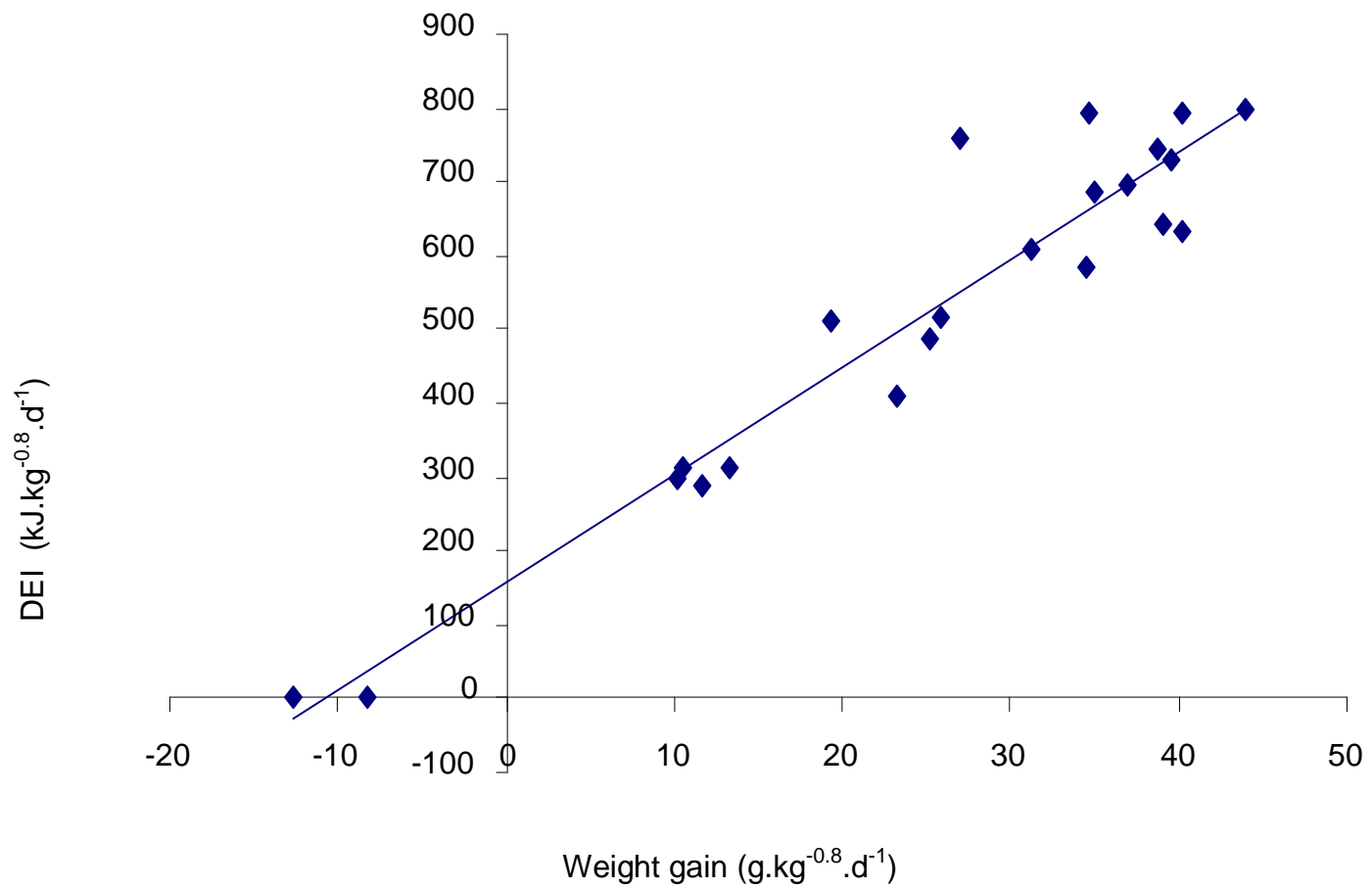


Fig. 2.4. The relationship between daily digestible energy intake (DEI) and weight gain of Atlantic salmon at 19°C fed diets of different DP:DE (DEI (kJ.kg^{-0.8}.d⁻¹) = 14.64 (±1.03) Weight gain (kJ.kg^{-0.8}.d⁻¹) + 155.34 (±30.46) (R² = 0.91, F = 202.83, P = 0.000, n = 22)).

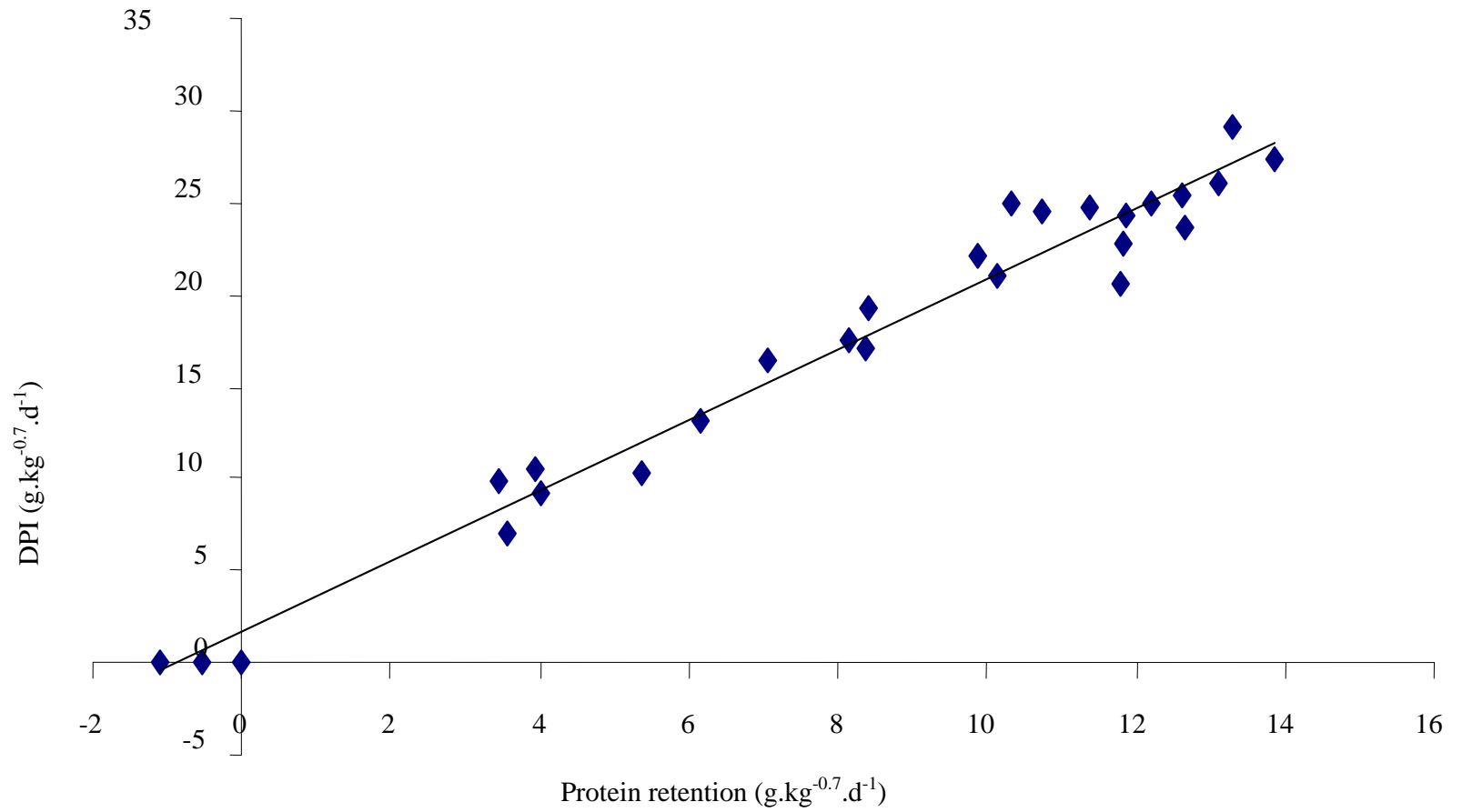


Fig. 2.5. The relationship between daily digestible protein intake (DPI) and protein retention of Atlantic salmon at 19°C fed diets of different DP:DE ($DPI (g.kg^{-0.7}.d^{-1}) = 1.87(0.08).Protein\ gain (g.kg^{-0.7}.d^{-1}) + 2.19(0.78)$ ($R^2 = 0.96$, $F = 514.9$, $P = 0.000$, $n = 22$)).

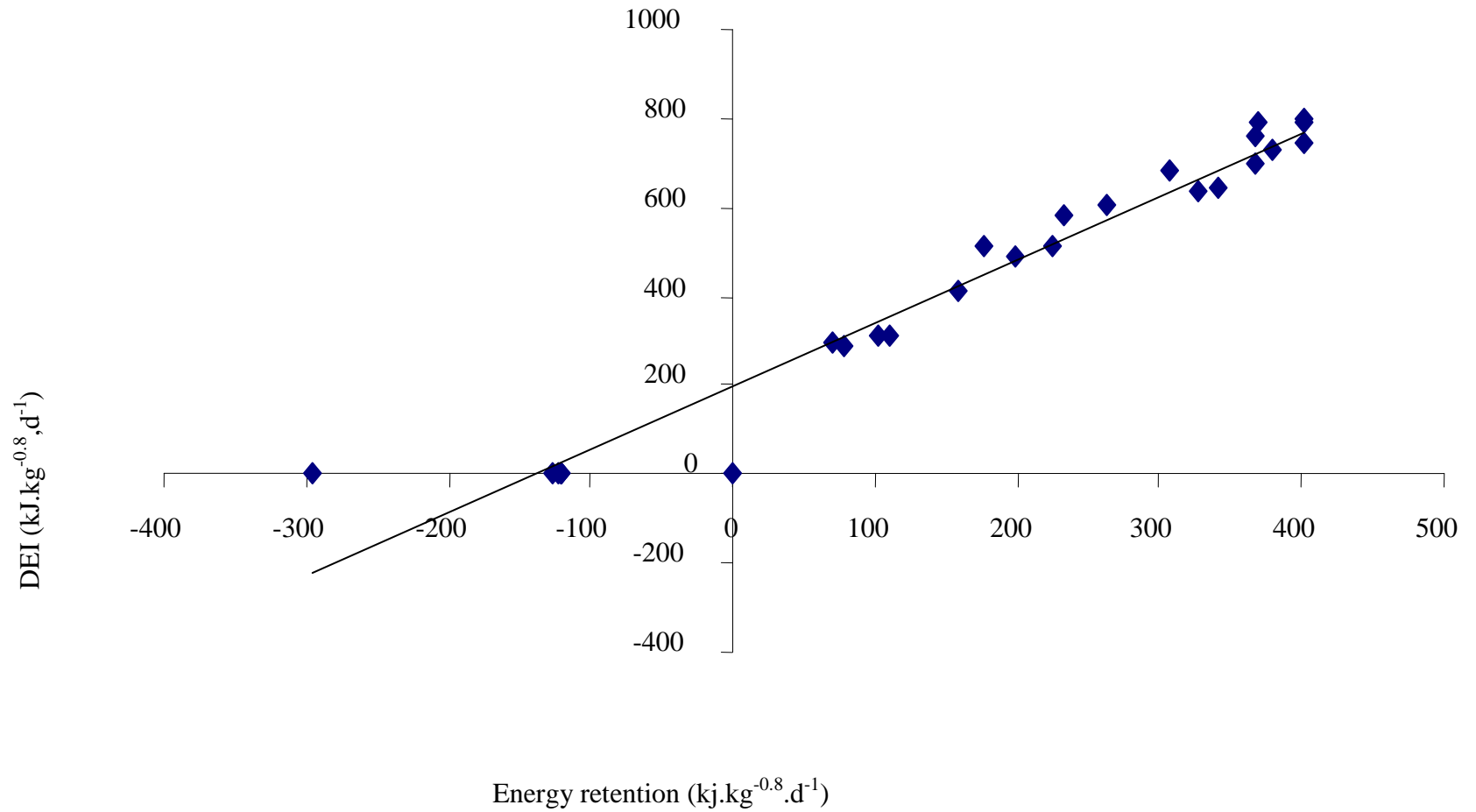


Fig. 2.6. The relationship between digestible energy intake (DEI) and energy retention of Atlantic salmon at 19°C fed diets of different DP:DE (DEI (kJ.kg^{-0.8}.d⁻¹) = 1.49 (±0.05) Energy gain (kJ.kg^{-0.8}.d⁻¹) + 185.77 (±12.96) (R² = 0.98, F = 1019.76, P = 0.000, n = 22)).

Chapter 3

Influence of dissolved oxygen on the optimum dietary protein to energy ratio for seawater Atlantic salmon, *Salmo salar* L., at an elevated temperature of 19°C**Abstract**

The effect of high (> 90% saturation) and low (~ 60% saturation) dissolved oxygen (DO) on optimum dietary digestible protein to digestible energy (DP:DE) ratio for seawater Atlantic salmon (*Salmo salar* L.), initial wet weight 105.1 g ± 1.9 g, was determined at an elevated water temperature of 19°C. Four extruded diets were produced on a commercial extruder and vacuum coated with fish oil. The diets were formulated to be either high (H) or low (L) in protein and energy: L:L (low protein and low energy); L:H (low protein and high energy); H:L (high protein and low energy) and H:H (high protein and high energy). Two diets, L:L and H:H, were fed to satiation at high DO. All diets were fed to satiation at low DO, H:H was also fed at 80, 60 and 30% of satiation at each dissolved oxygen level. There was a linear relationship between dietary digestible energy content and dry matter feed intake but not between dietary energy and digestible energy intake. DO had no effect on apparent digestibility whereas diet had a significant effect on both energy and nitrogen apparent digestibility. Whole body crude protein was not affected by diet or DO. Dry matter, lipid and energy content were not affected by diet but were significantly lower under low DO. Weight gain was significantly affected by DO and marginally by diet. Significant linear relationships were established between nutrient intake and growth for wet weight, protein and energy gain. Wet weight maintenance requirements were 4.92 g DP.kg^{-0.7}.d⁻¹ for dietary protein and 151.57 kJ DE.kg^{-0.8}.d⁻¹ for dietary energy. Protein and energy retention efficiency above maintenance were not affected by dietary DP:DE and were 55 and 70%, respectively. This research provides information for the optimisation of practical feeding of Atlantic salmon under more extreme summer conditions of high temperature and low dissolved oxygen.

3.1. Introduction

Temperature is a key determinant of growth and acts as a controlling factor that determines metabolic requirements for nutrients and governs the rate of physiological processes involved in nutrient utilisation (Brett, 1979). Environmental oxygen is a limiting factor that exerts its influence on growth depending on the complex interactions between the fish and the external conditions (Brett, 1979). At low dissolved oxygen (DO) growth increases with increasing DO concentration because oxygen becomes progressively less limiting. When DO concentration is no longer limiting growth then plateaus although at very high DO concentrations there may be a decrease in growth. When the effect of increasing temperature and DO concentration are considered together the key processes are the increase in oxygen (metabolic) demand by the fish and the decreasing oxygen concentration of water. Temperature and DO influence feed intake and this impacts on growth, whether low DO impacts directly on growth is less clear (Andrews et al., 1973; Brett, 1979).

At a practical level, oxygen concentration within sea-cages may also be less than 100% saturation and may vary considerably within the cage and over the daily cycle due to many influences such as tides, amount of sunlight and feeding regimes. Regardless of the reasons, the impact of oxygen concentration on fish performance will

largely depend on whether the DO concentration is below a threshold and therefore limiting, and if it is the extent of the limitation.

Clearly the effects of low dissolved oxygen become more pronounced and more critical at lower DO. Major effects relate to behavioural and physiological responses that reduce metabolic expenditure and include reduced activity and decreased appetite. The response of the fish, the severity and permanence of low DO effects will also depend on the length of time as well as how low oxygen concentration is. The focus of the current research was on moderately decreased DO that would be typical of routine events on a Tasmanian farm. Early research on salmonids determined thresholds for low DO affecting key feeding and growth parameters as well as survival. For example for coho salmon (*Oncorhynchus kisutch*) growth was zero at DO below 2.3 mg O₂ l⁻¹ and feed intake, growth and growth efficiency are affected below 5 mg O₂ l⁻¹ (Herrmann et al., 1962; Brett and Blackburn, 1981). Few published studies are available on the effects of moderately low DO on the optimum digestible protein to digestible energy (DP:DE) ratio in Atlantic salmon.

In Chapter 2, protein and energy utilisation of seawater Atlantic salmon (*Salmo salar* L.) was determined at an elevated temperature of 19°C and at a DO content of 100% saturation. The present experiment aimed to investigate the combined effect of elevated temperature and low DO on the energy and protein utilisation of seawater Atlantic salmon at an elevated temperature of 19°C. The experiment used the same diets and followed a similar organisation to that described in Chapter 2 in order to directly compare the two data sets. Four diets with combinations of high and low protein and energy were fed to satiation at normal and moderately low DO. Restricted rations were also used to obtain relationships between nutrient intake and growth. The critical information was to determine whether Atlantic salmon performed better on a different diet formulation at low DO than at a normal DO concentration. This information would contribute to feed management practices and selection of the most appropriate feeds depending on farm characteristics and local conditions.

3.2. Material and methods

3.2.1. Experimental diets

Four experimental diets were produced using a commercial extruder (Wenger X185 Optima) and vacuum-coated with fish oil (Skretting, Cambridge, Tasmania, Australia). The diets were formulated to be either high (H) or low (L) in protein and energy (Table 3.1): L:L low protein low energy; L:H low protein high energy; H:L high protein low energy; H:H high protein high energy. The protein was supplied from anchovy meal (Austral Group, Peru), soybean meal (Hunter Grain, NSW, Australia), corn gluten meal (Hunter Grain, NSW, Australia), feather meal (Camelleri Stockfeeds, NSW, Australia) and whole wheat (Cooperative Bulk Handling, WA, Australia). Lipid was supplied from anchovy oil (Sindicato Pesquero, Peru). A vitamin/mineral premix (commercial preparation according to NRC 1993 requirements for salmonids) was added. To measure digestibility an inert marker (Yb₂O₃; 0.5% DM (dry matter)) was mixed with half the fish oil, this was then used to coat the feed “kernel”. The remaining fish oil was then added on over this as a top coat to reduce the loss of ytterbium to the water. Feeds were stored at 1 – 2°C for the duration of the experiment.

3.2.2. Growth experiment

The experiment was conducted at the School of Aquaculture, University of Tasmania. Atlantic salmon (*Salmo salar* L.) smolt were obtained from Wayatinah Salmon Hatchery (SALTAS, Tasmania, Australia) and stocked in twenty-five 300-l tanks in a partial recirculation system (Carter and Hauler, 2000). The system was divided into two systems of 9 and 16 tanks in order to more easily manage the dissolved oxygen content in the low treatment tanks. The fish were acclimated to saltwater (30 ppt) over 6 days in daily increments of 5 ppt. The temperature was then steadily raised from 12°C to 19°C over 12 days. During acclimation fish were fed the H:H diet. Water was treated through physical and bio-filters with partial water changes as required to ensure water quality parameters (DO, pH, ammonia, nitrite and nitrate) stayed within the limits recommended for Atlantic salmon (Tarazona and Munoz, 1995).

At the start of the experiment, fish were anaesthetised (50 mg l⁻¹, Benzocaine) and weight and fork length measured. Fish less than 60 g, more than 200 g or with damage/disability were not used. Nine fish were randomly allocated to each of 24 300-l tanks to ensure there were no significant differences between group mean weights (105.1 ± 1.9 g). Ten additional fish were killed for the assessment of initial whole body chemical composition (see below). As noted above 16 tanks were allocated to the low DO treatment and run as a separate system from the remaining 9 tanks. Within each oxygen treatment, the tanks were randomly assigned to a diet and ration. For the high DO treatment two diets, L:L and H:H, were fed to satiation in triplicate. H:H was also fed at three restricted ration levels of 80%, 60% and 30% satiation. For the low DO treatment, all four diets were fed in triplicate to satiation, the H:H diet was also fed at each of the three restricted ration levels.

The dissolved oxygen content of the tanks was monitored twice daily, just before each feeding session (HACH HQ10 Portable LDO Dissolved Oxygen Meter). The dissolved oxygen content of the water in the tanks of the low DO treatment was slowly lowered over the first 2 weeks of the experiment by removing the air-stones and reducing the water flow. The target for the high DO treatment was 100% saturation (7.6 mg O₂.l⁻¹) and 70% saturation (5.6 mg O₂.l⁻¹) for the low DO treatment.

The fish were fed at 0900 and 1600 every day and feed intake determined using collectors that were placed over each tank's water outlet. The extruded pellets remained intact prior to collection and uneaten feed was estimated from the number of leftover pellets using the average weight of a pellet for each feed (Helland et al., 1996). Feed supplied to the satiation tanks was always in excess. The restricted rations were calculated from the previous day's average measured feed intake at satiation for that diet. The fish were re-weighed every 21 days and the experiment continued for 104 days, by which time the satiation-fed fish had more than doubled in weight. Prior to the end of the experiment the fish were not fed for a day. All fish were anaesthetised and weight and fork length measured. Fish were euthanised by an overdose of anaesthetic (benzocaine) and taken for analysis of whole body chemical composition (see below). All fish were anaesthetised and weights and fork length measured and three fish of good condition factor were killed for determination of whole body chemical composition determination.

All calculations were based on tank means from the satiation treatments. Specific growth rate (SGR) was calculated as $SGR (\% d^{-1}) = 100((\ln(W_f/W_i)) / t)$, where W_i and W_f were the initial and final wet weights (g) and t the number of days. Feed, protein and energy efficiency ratios (FER, PER, EER) were calculated at the end of the experiment as $ER (g.g^{-1} \text{ or } g.kJ^{-1}) = ((W_2 - W_1) / N_{intake})$ where W_2 and W_1 were the wet weights (g) at two times and N_{intake} was nutrient intake of feed (g), protein (g) or energy (kJ). Digestible protein and digestible energy efficiency ratios (DPER and DEER) were calculated using the above formula, except that N_{intake} was the digestible nutrient value. The efficiency with which consumed protein and energy were retained was calculated as productive protein value (PPV) and productive energy value (PEV) so that $PV (\%) = 100((N_f - N_i) / N_{intake})$ where N_f and N_i are the final and initial amounts of the nutrients in the fish and N_{intake} was the amount of the nutrient consumed by the fish. The digestible PVs (DPPV and DPEV) were also calculated using the above formula and digestible nutrient values.

3.2.3. Apparent digestibility

Apparent digestibility (AD, %) was measured at the end of the experiment using all tanks in the growth trial. The fish were fed the ytterbium-coated feeds for 8 days and for the last 6 days faecal samples were collected by settlement using collectors attached to the tanks between 1700 and 0900h (Carter and Hauler, 2000; Ward et al., 2005). The samples were freeze-dried, ground with a mortar and pestle and fish scales removed. Feed samples were also freeze-dried and ground. These samples were used in the analysis of nutrients and ytterbium marker (see below). Apparent digestibility was calculated as $AD (\%) = 100 - (100 (\%I_{diet} / \%I_{faeces}) \times (\%N_{faeces} / \%N_{diet}))$ (Maynard and Loosli, 1969) where I is the inert marker and N the nutrient in the diet and faeces. The digestible protein and energy values were applied to the crude protein and gross energy data to determine the digestible protein (DP) and digestible energy (DE) content of the 4 diets.

3.2.4. Chemical analysis

Standard methods were used to determine dry matter (freeze dried to constant weight); nitrogen (Kjeldahl using a copper catalyst); total lipid (Bligh and Dyer, 1959); energy (bomb calorimeter: Gallenkamp Autobomb, calibrated with benzoic acid); ash (AOAC, 1995). Ytterbium concentrations in the feed and faeces were determined using Flame Atomic Absorption Spectrometry (Varian AAS) (Ward et al., 2005).

3.2.5. Statistical analysis

Data are reported mean \pm SEM (standard error of the means). Comparison between means from satiation treatments ($n=3$) was by one-way ANOVA, with post-hoc analysis by Tukey. The slopes of the regression lines were compared by ANCOVA ($n = 5$); where there was no significant difference between treatments the data were pooled. Significance was accepted at probabilities of < 0.05 . Analyses were carried out with SPSS for Windows.

3.3. Results

3.3.1. Dissolved oxygen and temperature

The average dissolved oxygen concentrations for the high and low DO treatments, respectively, were 92.6% (7.1 mg O₂ l⁻¹) and 73.2% (5.6 mg O₂ l⁻¹) of saturation. Although the target DO was met in the low DO treatment there was some day to day variation and an initial period, when the systems were being adjusted, during which DO was higher than the target (Fig. 3.1). When fish died in the low DO tanks, dissolved oxygen increased in that tank, due to the lower mass of fish, until water flow was adjusted accordingly. The temperature of the low DO treatment was initially lower than that of the high DO treatment, although this was only by a maximum of 0.8°C (Fig. 3.2)

3.3.2. Digestible nutrient intake

The apparent digestibility (AD) of protein, lipid and energy were more than 89, 94 and 88%, respectively (Table 3.2). There was no interaction between DO and diet and the effects of DO and diet were analysed. DO had no effect on AD whereas diet had a significant effect on both AD_N and AD_{KJ} (Table 3.2). Feed intake at satiation was influenced by diet and by DO, it was significantly higher at the high DO and for L:L diet which had significantly higher intake than L:H and H:L (Table 3.3). There was a significant inverse relationship between dietary digestible energy (DE) content and feed intake (FI) which meant that there was no relationship between dietary DE and DE intake (Fig. 3.3). Fish on the lower energy diets ate more than those on diets of higher energy diets so that total digestible energy intake (DEI) was not significantly different between diets. The dietary digestible protein content did not affect feed intake or digestible protein intake (Fig. 3.4).

3.3.3. Growth and whole body chemical composition

Mortalities were 10% or less with no significant difference between treatments. Fish grew consistently over the course of the experiment, with wet weight at least doubling in each treatment. Final weight, wet weight gain and SGR were significantly lower at low DO. Diet significantly affected wet weight gain and SGR, so that fish on the L:L diet grew significantly better than the L:H diet (Table 3.3). There were no significant interactions between DO and diet for the measures of whole body chemical composition (Table 3.4). Diet did not have a significant effect whereas DO had a significant effect on DM, total lipid, ash and gross energy. DM, total lipid and gross energy were higher at high DO, whereas ash was lower. Feed efficiency ratio (FER) was not significantly different between diets or DO and varied between 0.90 and 0.98 g.g⁻¹ (Table 3.5). Protein efficiency measured as PEV, DPEV and PPV was significantly affected by diet but not by DO. Protein efficiency was higher for L:L than H:H, which in turn was higher than for H:L. Energy efficiency was not affected by diet or DO.

3.3.4. Modelling

The relationships between nutrient intake and weight gain were investigated by combining the satiation and restricted feeding treatments for feed intake (Fig. 3.5),

digestible protein intake (Fig 3.6) and digestible energy intake (Fig 3.7). ANCOVA showed no significant difference between the slopes of the regression lines so the data were pooled to calculate one regression for each relationship, all of which were highly significant ($P < 0.0001$). Weight exponents of -0.7 and -0.8 were used with protein and energy data, respectively (see Chapter 2). Similarly, data for intake and retention in relation to protein (Fig 3.8) and energy (Fig 3.9) were combined.

Maintenance of wet weight required feed intake of $4.82 \text{ g kg}^{-0.8} \text{ d}^{-1}$ (Fig 3.5). For digestible intake of protein and energy the wet weight maintenance requirements were $4.06 \text{ g DP.g}^{-0.7} \text{ .d}^{-1}$ (Fig 3.6) and $117.77 \text{ kJ DE.g}^{-0.8} \text{ .d}^{-1}$ (Fig 3.7), respectively. Similarly, for digestible intake of protein and energy the protein and energy maintenance requirements were $3.39 \text{ g DP.g}^{-0.7} \text{ .d}^{-1}$ (Fig 3.8) and $147.63 \text{ kJ DE.g}^{-0.8} \text{ .d}^{-1}$ (Fig 3.9), respectively. The slope of the line represents the cost of growth, which was 1.81 and 1.43 for protein and energy, respectively. The reciprocal values ($1/1.81$ and $1/1.43$) calculated efficiencies of utilisation for growth above maintenance of 0.55 for digestible protein and of 0.70 for digestible energy.

3.4. Discussion

Studies on the effects of low dissolved oxygen (DO) on fish growth tend to investigate critical threshold concentrations for parameters such as survival, feed intake and growth. Fewer studies have examined the effects of smaller reductions in DO on growth that are relevant to summer farming conditions, for example in Tasmania where summer water temperatures can range from 17 to 21°C. At these temperatures oxygen concentration is unavoidably low due to the reduced solubility of oxygen with increasing temperature and DO can decrease to 60% saturation ($4.5 \text{ mg O}_2 \text{ l}^{-1}$) in commercial sea-cages. The current experiment used the same four diets as a previous experiment (Chapter 2) to investigate the effect of DO on Atlantic salmon growth in relation to dietary DP:DE at an elevated temperature. The influence of diet on growth has been discussed (Chapter 2) and the effect of reduced DO will be the main focus of this discussion. The combined data show essentially the same responses in the two experiments (Fig 3.10). Protein retention was slightly lower in the current experiment. In the low DO treatment the average DO concentration was $5.6 \text{ mg O}_2 \text{ l}^{-1}$ compared to $7.1 \text{ mg O}_2 \text{ l}^{-1}$ in the high DO treatment.

3.4.1. Digestibility and nutrient intake

Low DO did not affect apparent digestibility of crude protein, lipid or gross energy. The effect of diet on apparent digestibility of protein in the current experiment was similar to that in Chapter 2 and protein digestibility was positively related to dietary protein content. Crude lipid was well digested and not influenced by diet. DO in the low treatment was not low enough to affect digestibility. At DO of $4\text{-}6 \text{ mg O}_2 \text{ l}^{-1}$ (40% saturation) apparent digestibility was not affected (Pedersen, 1987; Pouliot and De la Noue, 1988).

Atlantic salmon regulated feed intake to meet their energy requirement and digestible energy intake showed a positive linear relationship with dietary digestible energy content in this experiment as in the previous experiment (Chapter 2). Hence DO did not affect this relationship and the slopes were similar for both experiments. However, DO did affect feed intake and it was significantly lower under low DO.

While temperature is a main environmental factor influencing feed intake DO acts as a limiting factor (Andrews et al., 1973; Brett, 1979). Feeding increases oxygen consumption in fish and when oxygen is limited feed intake decreases as a behavioural response to reduce oxygen demand associated with feeding (Brett, 1979). For the salmonids rainbow trout and coho salmon appetite was suppressed when DO decreased to around 4-5 mg O₂ l⁻¹ (Herrmann et al., 1962; Medale et al., 1987; Pedersen, 1987). There appears to be a threshold of about 70% saturation for rainbow trout in freshwater (Pedersen, 1987). Decreased feed intake is likely to be a mechanism to survive chronic hypoxia by decreasing energy demand due to digestion and assimilation of nutrients (Kramer, 1987).

3.4.2. Growth and growth efficiency

In the current experiment growth was negatively affected by low DO. Hypotheses to explain why growth is reduced at lower DO include an increased energetic cost for the increased ventilation required to increase oxygen uptake across the gills and decreased appetite and feed intake in order to reduce oxygen demand (Brett, 1979; Kramer, 1987). Reduced nutrient intake and increased energy expenditure will both clearly reduce the nutrients available for growth.

The DO concentration at which growth is negatively affected varies with species. Yearling Coho salmon at 18°C survived for 30 days at a DO of 2 mg O₂ l⁻¹ although feed intake was very low and the fish lost weight: at 2.9 mg O₂ l⁻¹ the fish grew but less than at 9 mg O₂ l⁻¹ (Brett and Blackburn, 1981). In salmonid species, Coho salmon and sockeye salmon, the threshold DO, above which there are no adverse effects on growth, appears to range depending on the study so that concentrations from 4-5 mg O₂ l⁻¹ (Brett and Blackburn, 1981) to 7 mg O₂ l⁻¹ (Pedersen, 1987) have been noted. The low DO concentration in the current experiment was in the range of these values and clearly affected growth through the decreased feed intake. The adverse effect of low DO was mainly explained by decreased feed intake and the linear relationships between feed intake and growth showed that at a similar feed intake the growth rate and growth efficiencies of the Atlantic salmon were not different between normal and low DO. Similarly there were no significant difference in weight gain between European sea bass and turbot fed the same rations under hypoxic and normoxic conditions (Pichavant et al., 2001). Decreased growth efficiency has been reported in some studies including juvenile largemouth bass (Stewart et al., 1967), rainbow trout (Pedersen, 1987) and Atlantic cod (Chabot and Dutil, 1999). This can partly be accounted for by the proportionally higher maintenance requirement at lower feed intake, at a lower feed intake a large proportion of the nutrients in a meal are required to meet maintenance requirements and this leaves less available for growth. The experiments that match feed intake show there is no change in maintenance or growth requirements at different DO. Fish are also responsive to DO and will change their behaviour depending on how low DO is, a reduction in feed intake has been noted and there may be other changes such as reduced activity that will impact on energy expenditure and the nutrients available for growth.

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Table 3.1
Ingredient and chemical composition of experimental feeds

	Diet			
	L:L	L:H	H:L	H:H
Ingredient composition (g kg ⁻¹)				
Fish meal	427	427	534	480
Corn gluten	53	53	67	60
High Pro Soy (48%)	82	82	102	92
Feather meal	44	44	56	50
Wheat	150	149	111	130
Vit/Min Premix	3	3	3	3
Fish oil	141	242	127	185
Bentonite	100	0	0	0
Chemical composition (g kg ⁻¹ DM)				
Dry matter (g kg ⁻¹)	93.03	92.65	91.35	94.05
Crude protein (g kg ⁻¹)	410.51	418.21	433.66	449.41
Total lipid (g kg ⁻¹)	291.19	351.06	311.09	312.46
Total ash (g kg ⁻¹)	131.13	95.26	112.28	89.89
Gross energy (MJ kg ⁻¹ DM)	21.71	24.16	22.92	23.50
Digestible protein (g DP.kg ⁻¹ DM)	393.01	399.66	419.89	422.37
Digestible energy (MJ DE.kg ⁻¹ DM)	19.91	21.80	19.76	21.50
DP:DE (g DP.MJ ⁻¹ DE)	19.74	17.14	21.25	19.65

Table 3.2

Apparent digestibility (AD%) for Atlantic salmon at 19°C and at high (7.6 mg.l⁻¹) or low (5.6 mg.l⁻¹) dissolved oxygen (DO) fed diets with low and high protein and energy

		DO	High		Low			DO	Diet		DO x Diet			
		Diet	L:L	H:H	L:L	L:H	H:L	F	P	F	P	F	P	
AD _N	(%)		90.02 (0.51)	90.88 (0.35)	89.32 (0.27)	90.33 (0.18)	91.09 (0.48)	90.84 (0.35)	0.98	0.340	5.05	0.02	0.78	0.39
AD _L	(%)		94.06 (0.45)	94.33 (0.24)	94.13 (0.28)	94.44 (0.26)	94.81 (0.50)	94.33 (0.24)	0.01	0.920	0.81	0.51	0.01	0.92
AD _{kJ}	(%)		88.30 (0.23)	89.99 (0.44)	88.38 (0.29)	88.35 (0.29)	89.64 (0.08)	89.95 (0.16)	0.01	0.950	16.74	0.00	0.06	0.82

Mean ± SEM (n = 3).

Diets: L:L (low protein:low energy), L:H (low protein:high energy), H:L (high protein:low energy) and H:H (high protein:high energy).

AD for crude protein (N), lipid (L) and energy (kJ)

Table 3.3
Growth performance of Atlantic salmon at 19°C and high (7.6 mg.l⁻¹) or low (5.6 mg.l⁻¹) dissolved oxygen (DO) fed diets with low and high protein and energy

	DO Diet	High		Low				DO		Diet		DO x Diet	
		L:L	H:H	L:L	L:H	H:L	H:H	F	P	F	P	F	P
Initial weight	(g)	94.76 (1.72)	104.89 (3.39)	102.24 (4.53)	106.21 (3.84)	110.83 (8.25)	107.54 (9.56)	0.75	0.400	0.82	0.510	0.17	0.690
Final weight	(g)	282.40 (16.28)	280.09 (8.57)	252.01 (14.13)	205.2 (3.40)	212.79 (17.62)	213.02 (13.61)	13.32	0.003	1.86	0.190	1.89	0.195
Weight gain	(g)	187.63 (15.52)	175.20 (10.41)	149.77 (10.69)	98.99 (6.41)	101.96 (11.12)	105.48 (15.29)	19.93	0.001	3.50	0.050	1.75	0.210
SGR	(% d ⁻¹)	1.25 (0.06)	1.13 (0.06)	1.04 (0.04)	0.90 (0.05)	0.97 (0.04)	0.99 (0.12)	17.04	0.001	4.32	0.030	0.84	0.380
Feed intake	(g)	191.49 (13.39)	176.82 (7.49)	154.02 (7.33)	102.90 (4.86)	113.41 (8.08)	108.96 (10.56)	34.56	0.000	6.16	0.010	2.88	0.120

Mean ± SEM (n = 3).

Diets: L:L (low protein:low energy), L:H (low protein:high energy), H:L (high protein:low energy) and H:H (high protein:high energy).

Table 3.4

Whole body chemical composition of Atlantic salmon at 19°C and high (7.6 mg.l⁻¹) or low (5.6 mg.l⁻¹) dissolved oxygen (DO) fed diets with low and high protein and energy

	DO Diet	High		Low				DO		Diet		DO x Diet	
		L:L	H:H	L:L	L:H	H:L	H:H	F	P	F	P	F	P
Dry matter	(%)	35.00 (0.15)	34.74 (0.09)	32.65 (0.69)	34.69 (0.36)	33.74 (0.28)	33.83 (1.29)	20.28	0.001	0.59	0.630	0.91	0.360
Crude protein	(%)	17.90 (0.19)	17.78 (0.16)	17.47 (0.24)	17.85 (0.25)	17.58 (0.40)	17.82 (0.17)	0.05	0.990	0.85	0.490	0.01	0.100
Total lipid	(%)	14.99 (0.05)	15.50 (0.25)	13.66 (0.36)	14.71 (0.25)	13.30 (0.43)	13.20 (1.65)	21.13	0.001	3.13	0.070	1.04	0.330
Ash	(%)	2.14 (0.04)	2.25 (0.13)	2.43 (0.07)	2.35 (0.07)	2.24 (0.12)	2.43 (0.120)	6.19	0.030	1.05	0.410	0.30	0.600
Gross energy	(kJ.g ⁻¹)	8.76 (0.08)	8.67 (0.15)	8.36 (0.15)	8.82 (0.20)	8.16 (0.15)	8.08 (0.46)	12.25	0.004	3.13	0.070	1.95	0.190
Protein energy	(kJ.g ⁻¹)	4.23 (0.04)	4.21 (0.04)	4.13 (0.07)	4.22 (0.07)	4.16 (0.12)	4.31 (0.12)	0.00	0.980	0.85	0.490	3.12	0.100
Non-protein energy	(kJ.g ⁻¹)	4.53 (0.08)	4.67 (0.15)	3.83 (0.20)	4.60 (0.25)	4.00 (0.13)	3.77 (0.50)	0.06	0.904	2.70	0.090	0.36	0.560

Mean ± SEM (n =3).

Diets: L:L (low protein:low energy), L:H (low protein:high energy), H:L (high protein:low energy) and H:H (high protein:high energy).

Table 3.5
Nutrient efficiencies of Atlantic salmon at 19°C and high (7.6 mg.l⁻¹) or low (5.6 mg.l⁻¹) dissolved oxygen (DO) fed diets with low and high protein and energy

	DO Diet	High		Low				DO		Diet		DO x Diet	
		L:L	H:H	L:L	L:H	H:L	H:H	F	P	F	P	F	P
FER	(g g ⁻¹)	0.98 (0.03)	0.99 (0.03)	0.97 (0.03)	0.90 (0.2)	0.90 (0.03)	0.96 (0.05)	0.23	0.640	0.55	0.660	0.08	0.780
PPV	(%)	0.43 (<0.01)	0.39 (0.16)	0.53a (0.01)	0.45ab (0.01)	0.41b (0.03)	0.42ab (0.02)	0.45	0.520	4.47	0.030	1.26	0.280
PEV	(%)	0.48 (0.02)	0.46 (0.02)	0.43 (0.02)	0.49 (0.01)	0.45 (0.04)	0.46 (0.05)	0.89	0.360	0.64	0.600	0.44	0.520
PER	(g.g ⁻¹)	2.37 (0.06)	2.17 (0.06)	2.35a (0.08)	2.31ab (0.05)	1.92c (0.13)	2.12b (0.12)	0.24	0.640	5.50	0.010	0.07	0.790
EER	(g.MJ ⁻¹)	44.66 (1.21)	42.84 (1.13)	44.71 (1.49)	39.62 (0.91)	39.88 (2.62)	41.53 (2.27)	0.13	0.730	1.80	0.200	0.15	0.710
DPER	(g.g ⁻¹)	2.63 (0.09)	2.39 (0.06)	2.60a (0.09)	2.53ab (0.06)	2.13c (0.13)	2.32b (0.13)	0.14	0.720	6.33	0.010	0.12	0.730
DEER	(g.MJ ⁻¹)	50.59 (1.50)	47.60 (1.21)	50.60 (1.84)	44.84 (0.91)	44.50 (2.95)	46.17 (2.49)	0.13	0.730	2.20	0.140	0.13	0.720

Mean ± SEM (n =3).

Diets: L:L (low protein:low energy), L:H (low protein:high energy), H:L (high protein:low energy) and H:H (high protein:high energy).

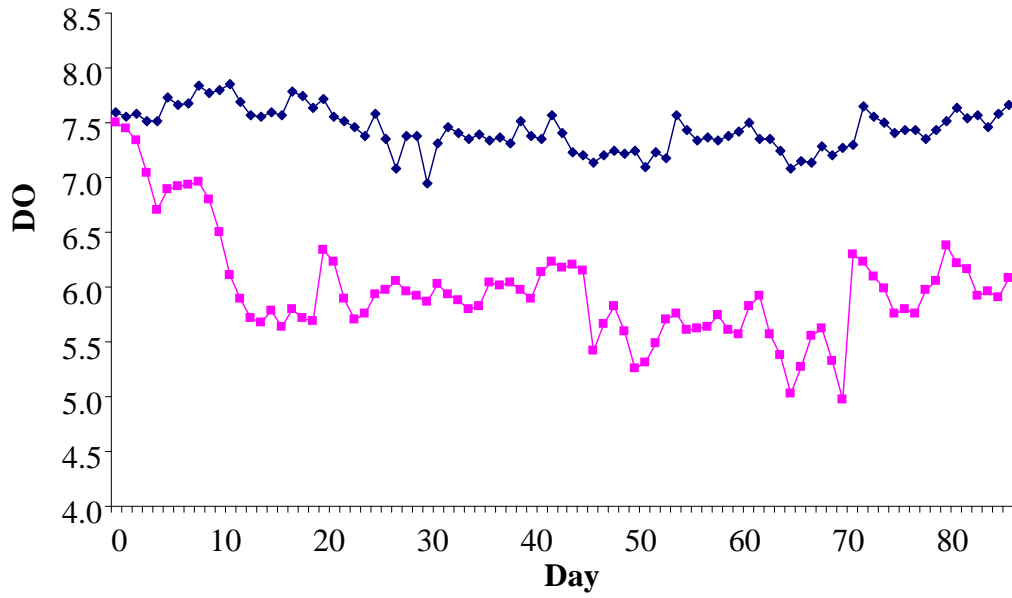


Fig. 3.1. Daily dissolved oxygen (mg O₂.l⁻¹) of high (♦ 7.6 mg.l⁻¹, n = 9) and low (■ 5.6 mg.l⁻¹, n = 15) treatments at 19°C.

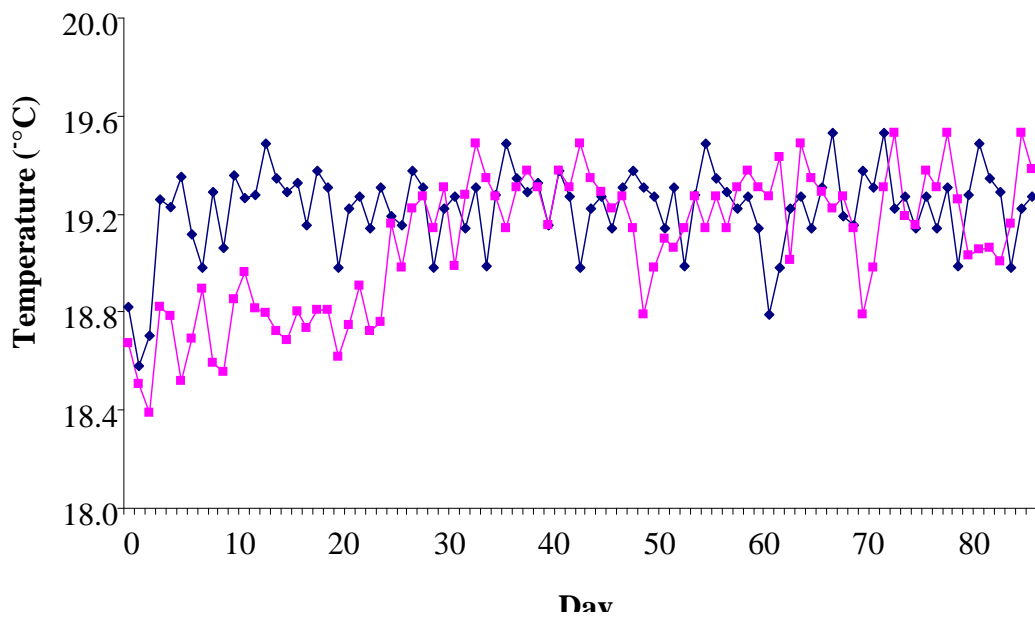


Fig. 3.2. Daily temperature of high (♦ 7.6 mg.l⁻¹, n = 9) and low (■ 5.6 mg.l⁻¹, n = 15) treatments at 19°C.

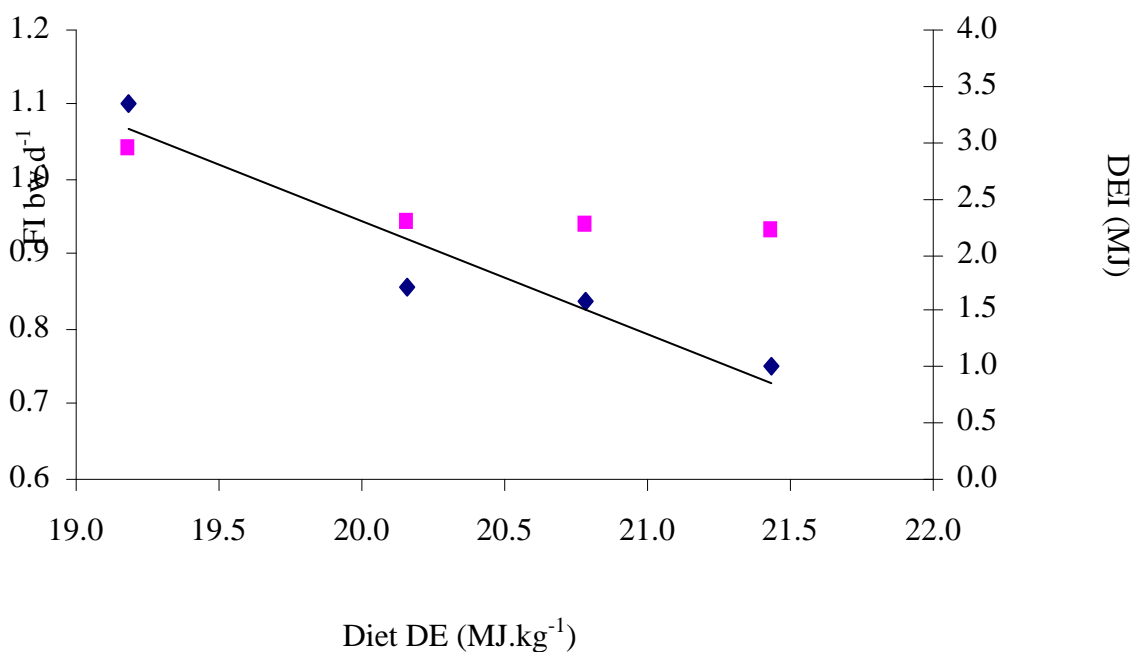


Fig. 3.3. Feed intake (FI%bw.d⁻¹; ◆) and digestible energy intake (DEI; ■) relative to dietary digestible energy content of the four experimental diets at 19°C and a low dissolved oxygen concentration (5.6 mg.l⁻¹): $FI (\%bw.d^{-1}) = 3.973 (\pm 0.53) - 0.151 (\pm 0.04) DE$ ($R^2 = 0.91$, $F = 36.04$, $P = 0.05$, $n = 4$).

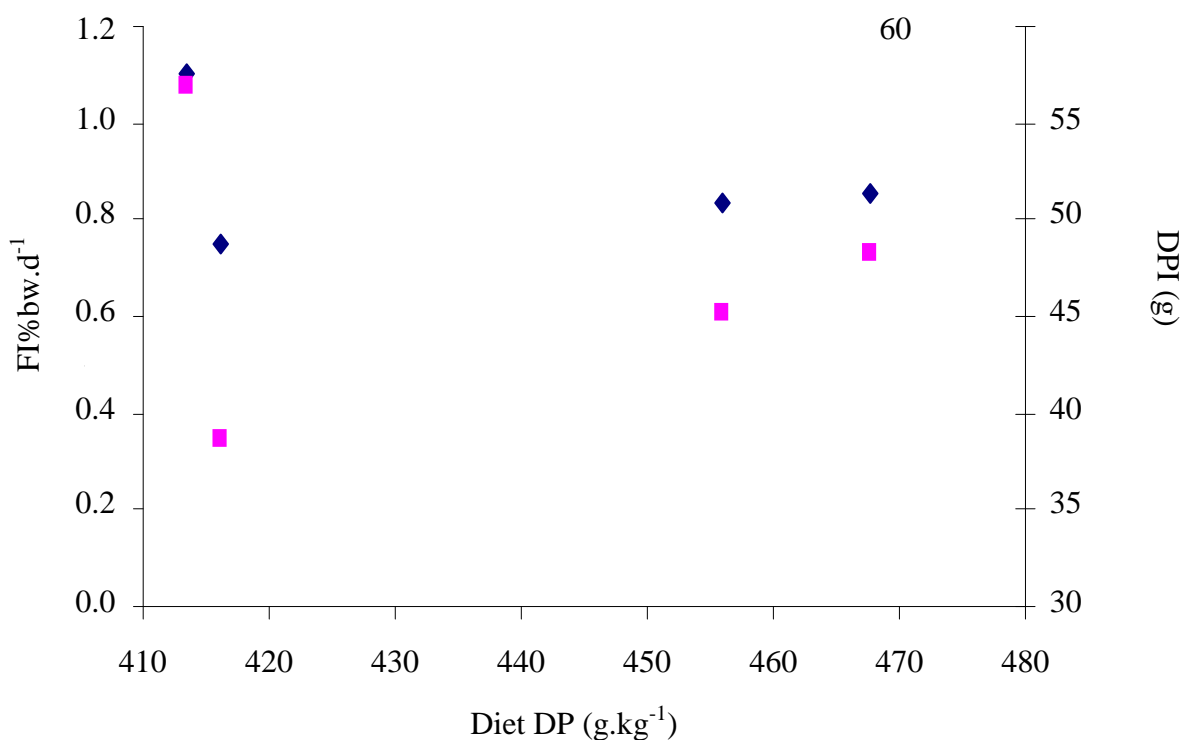


Fig 3.4. Feed intake (FI%bw.d⁻¹; ◆) and digestible protein intake (DPI; ■) relative to dietary digestible protein content of the four experimental diets at 19°C and dissolved oxygen level 5.6 mg.l⁻¹.

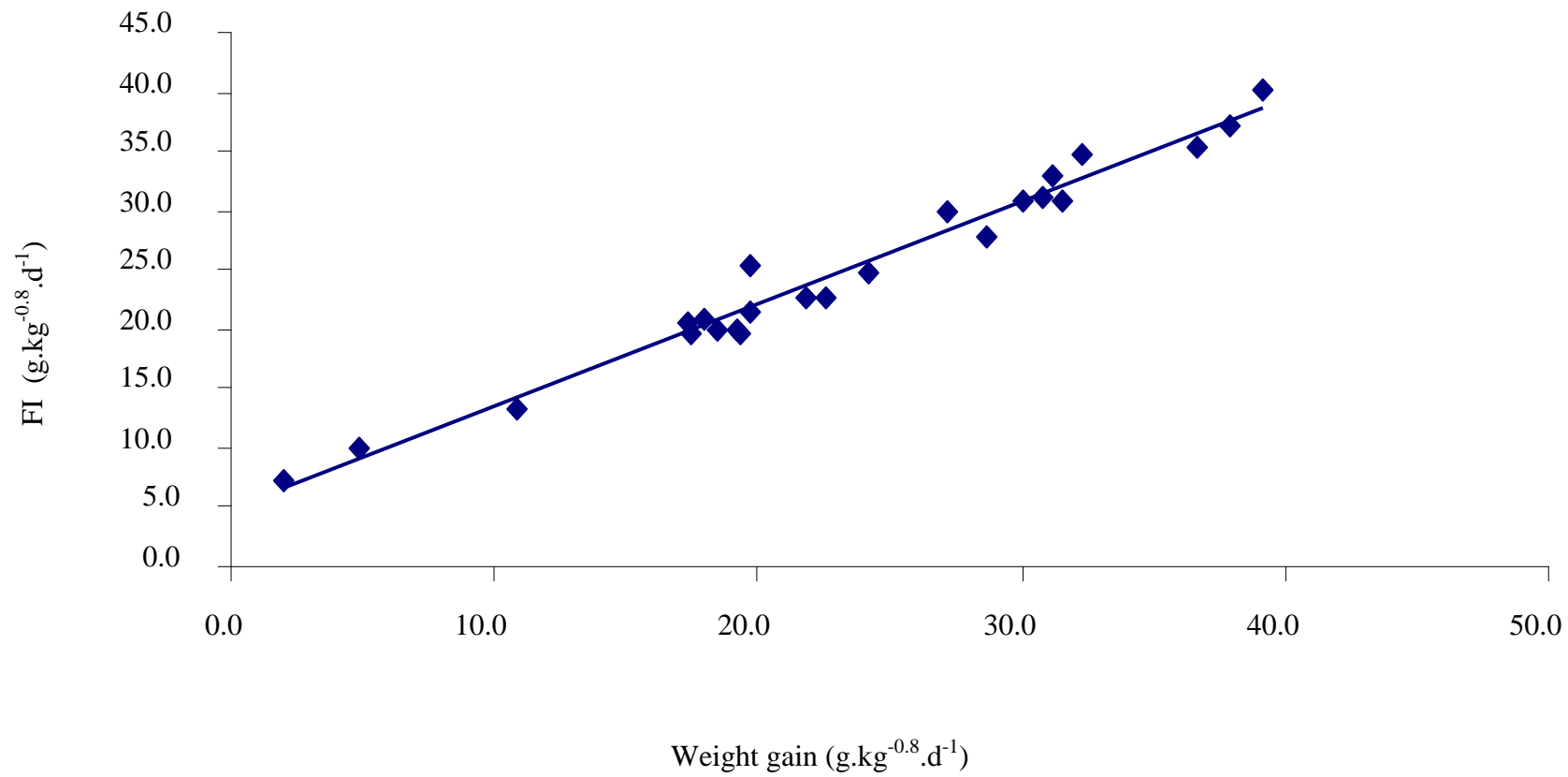


Fig. 3.5. The relationship between feed intake (FI, g.kg^{-0.8}.d⁻¹) and weight gain (g.kg^{-0.8}.d⁻¹) of Atlantic salmon fed diets of different DP:DE at 19°C and high and low dissolved oxygen: $FI (g.kg^{-0.8}.d^{-1}) = 0.86(0.03).Weight\ gain (g.kg^{-0.8}.d^{-1}) + 4.82(0.75)$ ($R^2 = 0.97$, $F = 834.7$, $P = 0.000$, $n = 22$).

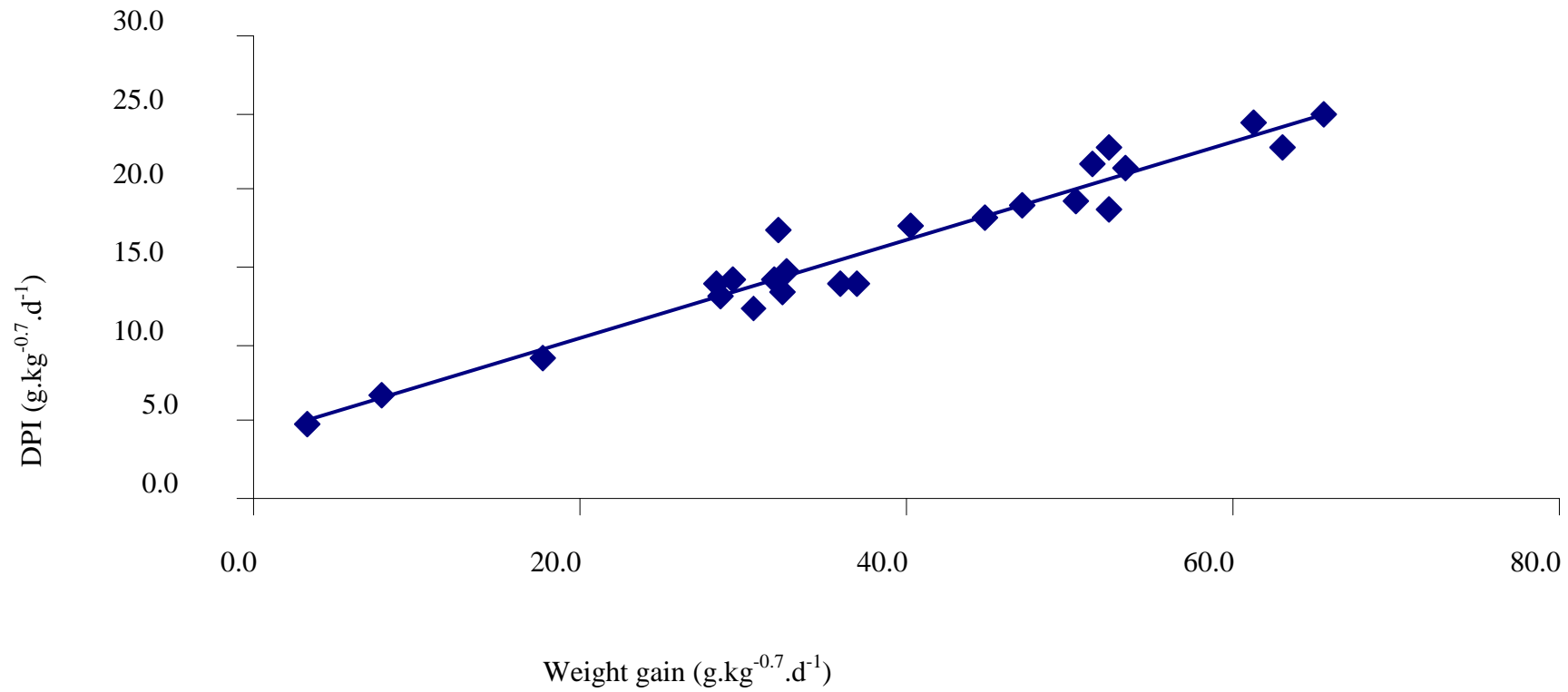


Fig. 3.6. The relationship between daily protein intake (DPI, g.kg^{-0.7}.d⁻¹) and weight gain (g.kg^{-0.7}.d⁻¹) of Atlantic salmon fed diets of different DP:DE at 19°C and high and low dissolved oxygen: $DPI (g.kg^{-0.7}.d^{-1}) = 0.32(0.02).Weight\ gain (g.kg^{-0.7}.d^{-1}) + 4.06(0.67)$ ($R^2 = 0.94$, $F = 389.9$, $P = 0.000$, $n = 22$).

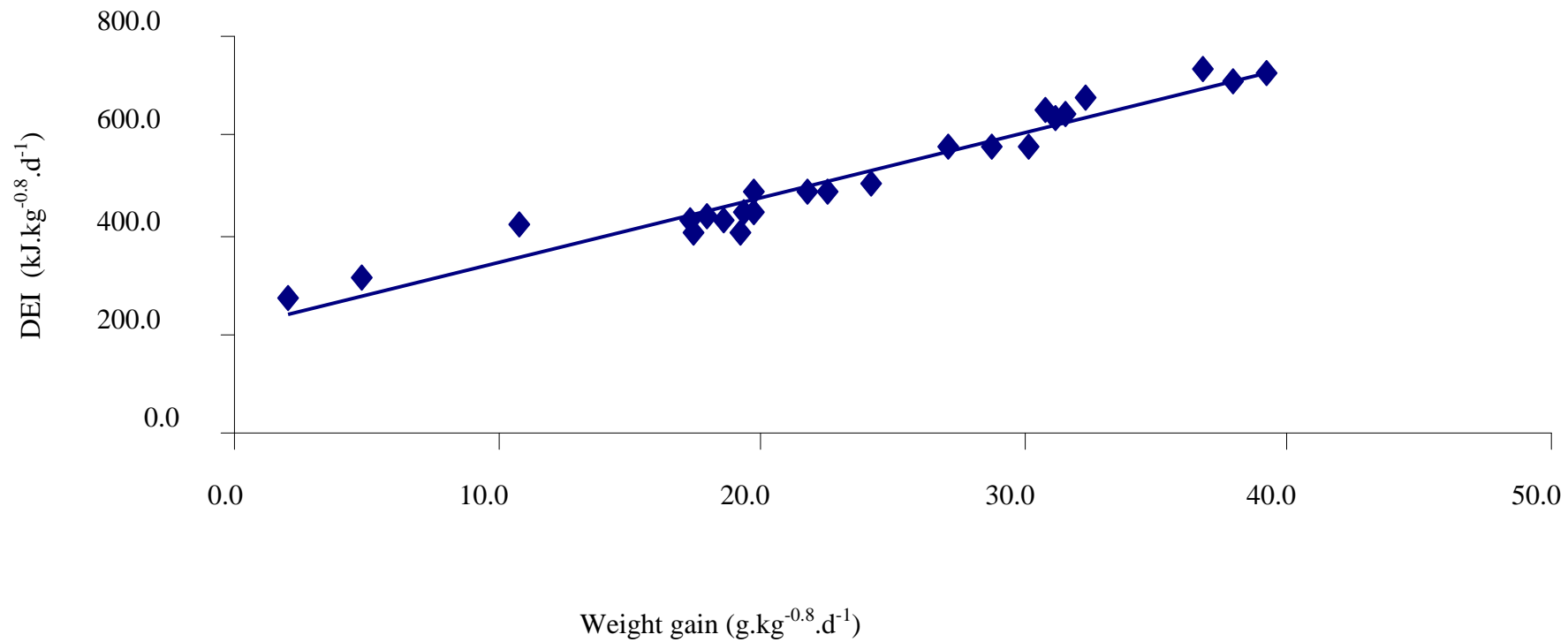


Fig. 3.7. The relationship between daily energy intake (DEI, kJ.g^{-0.8}.d⁻¹) and weight gain (kJ.g^{-0.8}.d⁻¹) of Atlantic salmon fed diets of different DP:DE at 19°C and high and low dissolved oxygen. $DEI (kJ.g^{-0.8}.d^{-1}) = 15.83(0.51).Weight\ gain (kJ.g^{-0.8}.d^{-1}) + 117.77(12.84)$ ($R^2 = 0.98$, $F = 1066.277$, $P = 0.000$, $n = 22$).

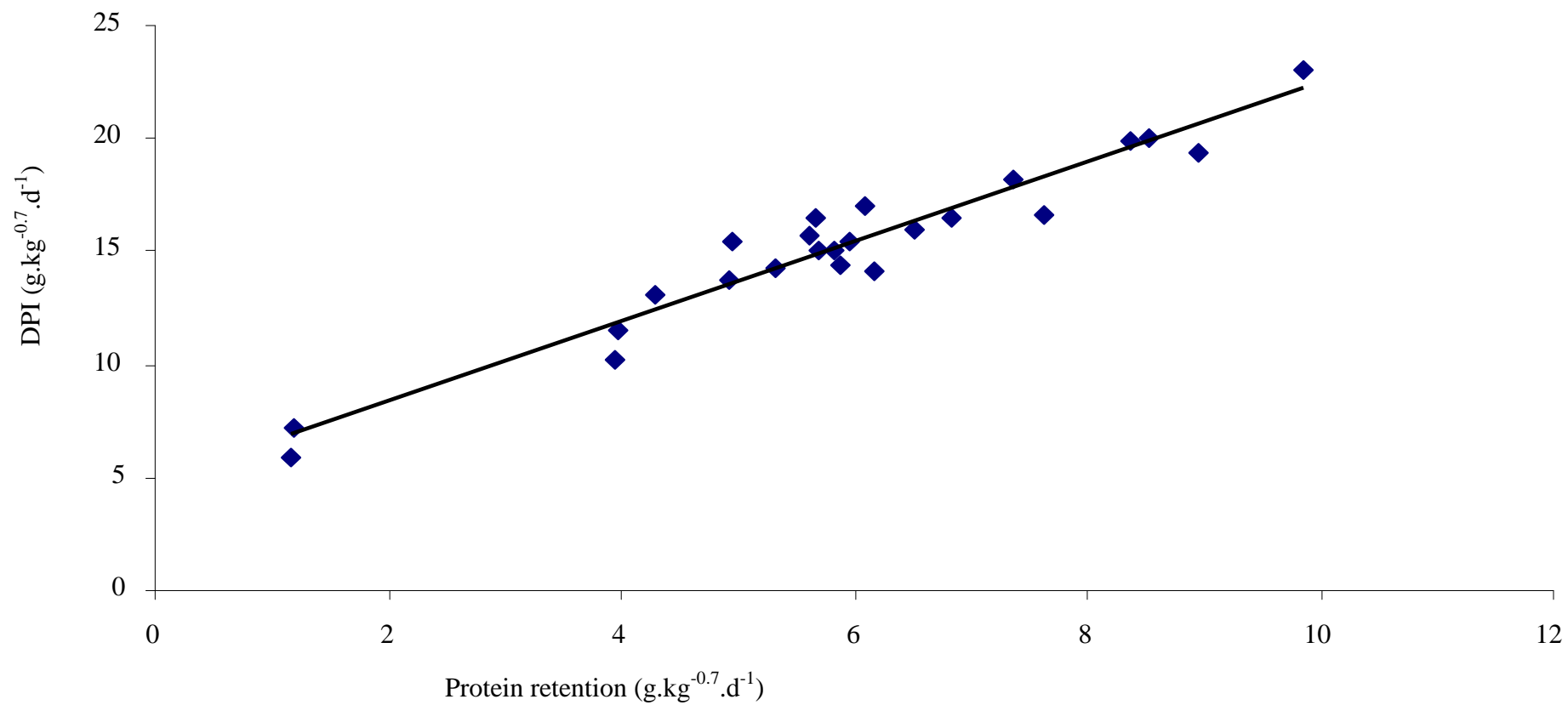


Fig. 3.8. The relationship between daily digestible protein intake (DPI, g.kg^{-0.7}.d⁻¹) and protein retention (g.kg^{-0.7}.d⁻¹) of Atlantic salmon fed diets of different DP:DE at 19°C and high and low dissolved oxygen. $DPI (g.kg^{-0.7}.d^{-1}) = 1.81(0.11).Protein\ retention (g.kg^{-0.7}.d^{-1}) + 3.39(0.84)$ ($R^2 = 0.92$, $F = 273.318$, $P = 0.000$, $n = 22$).

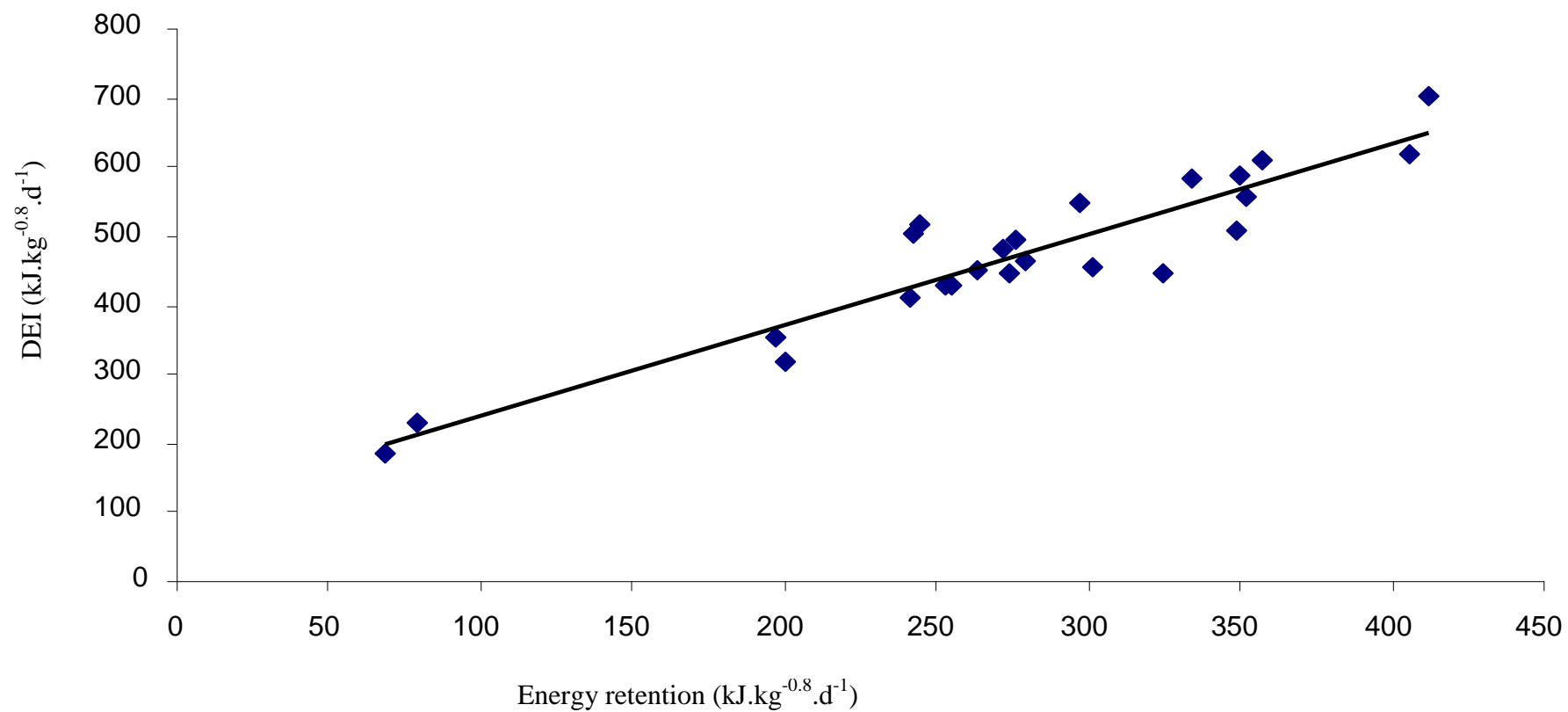


Fig. 3.9. The relationship between daily digestible energy intake (DEI, kJ.kg^{-0.8}.d⁻¹) and energy retention (kJ.kg^{-0.8}.d⁻¹) of Atlantic salmon fed diets of different DP:DE at 19°C and high and low dissolved oxygen. $DEI (kJ.kg^{-0.8}.d^{-1}) = 1.43(0.10).Energy\ retention (kJ.kg^{-0.8}.d^{-1}) + 147.63(25.99)$ ($R^2 = 0.91$, $F = 226.6$, $P = 0.000$, $n = 22$).

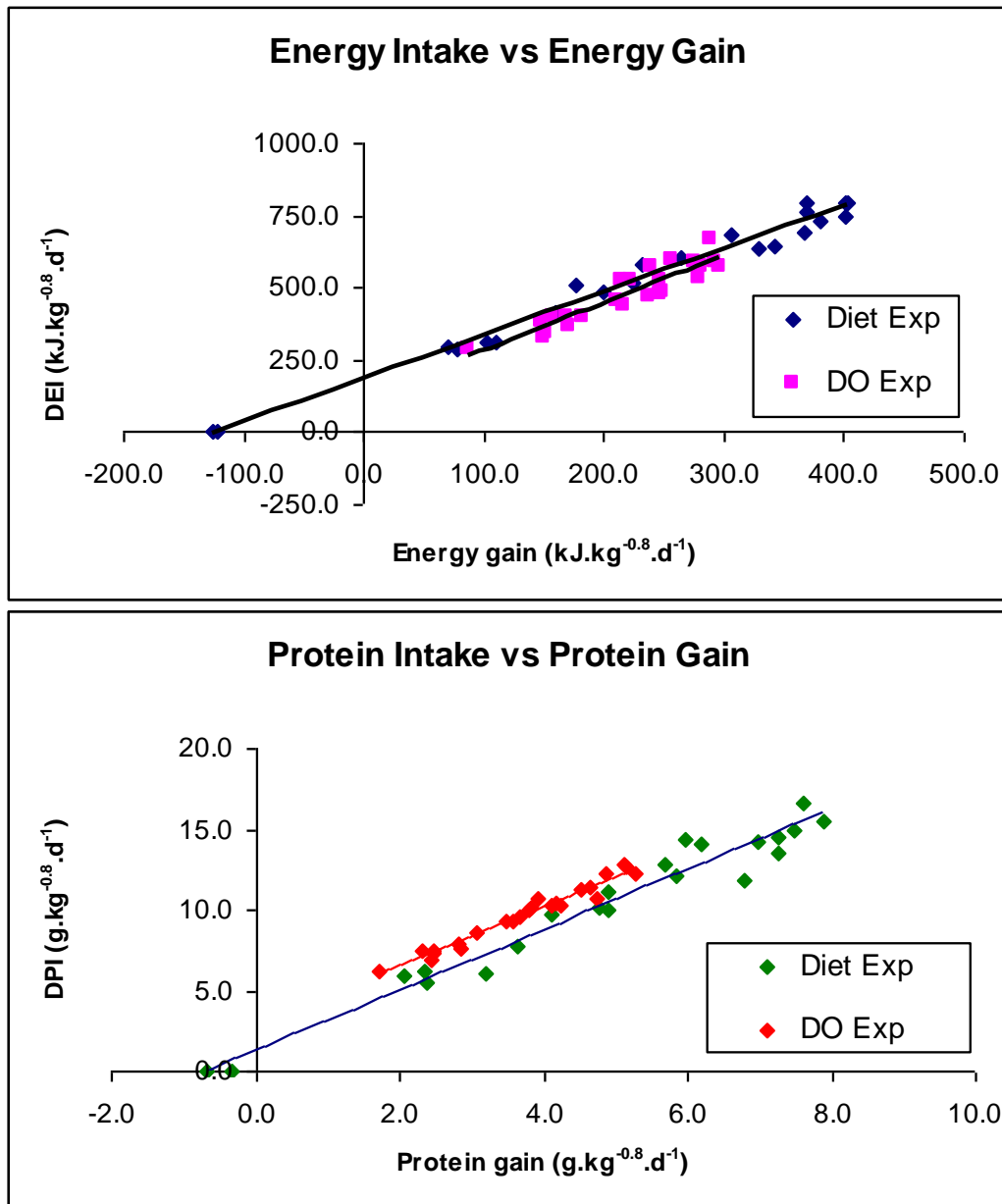


Fig. 3.10. The effect of low DO on the relationships between energy gain and digestible energy intake and b) the relationship between protein gain and digestible protein intake. The DO Exp line combines data from both the normal and low DO treatments.

Chapter 4

Maintenance requirements for seawater Atlantic salmon, *Salmo salar* L., at different temperatures**Abstract**

Two experiments were conducted in order to estimate the maintenance energy and protein requirements in seawater Atlantic salmon (*Salmo salar* L.) in relation to temperature. Using the change in whole body chemical composition following periods without feed was assessed as a straightforward method to provide maintenance estimates and to compare differences in protein and energy metabolism at 12, 16 and 18°C. Salmon with a relatively wide range of wet weights, 0.58–0.93, 0.50–1.77 and 0.60–0.92 kg at three temperatures, were used to provide data sets. Protein losses, weight corrected using an exponent of -0.7, at 12, 16 and 18°C were -0.498 (± 0.120), -0.490 (± 0.359) and -0.518 (± 0.110) g P kg^{-0.7} d⁻¹, respectively. Energy losses, weight corrected using an exponent of -0.8, were -26.04 (± 6.46), -29.22 (± 23.96) and -26.90 (± 5.61) kJ kg^{-0.8} d⁻¹, respectively. As expected, maintenance requirements calculated from losses of protein and energy were lower than calculated from ration-growth analysis. The measurement of changes in whole body chemical composition was not recommended as a straightforward (shortcut) method to calculate quantitative maintenance requirements. Information that can be obtained from starvation experiments was discussed, starvation experiments combined with physiological and molecular methods could potentially reveal important information. For example, there was some indication of an important temperature threshold between 18 and 19°C.

4.1. Introduction

The nature of the relationships between temperature and bioenergetic parameters, principally feed intake, growth and metabolism, has been well described for fish (Brett, 1979; Jobling, 1981; 1994). Increased water temperature, within the thermal tolerance of a species and up to the optimum temperature, drives increased metabolism, feed intake and growth (Jobling, 1981; 1994; McCarthy and Houlihan, 1996). Above the optimum temperature, growth and growth efficiency decrease due to feed intake failing to meet the exponential increase in metabolism and consequent increase in energy (nutrient) demand (Katersky and Carter, 2005). A component of the increased demand is to meet maintenance requirements and the aim of the present research was to determine whether nutrient losses due to starvation could be used to estimate the energy and protein maintenance requirements for Atlantic salmon at different temperatures. Several maintenance requirement values have been determined for salmonid species but there is little information available for large Atlantic salmon (*Salmo salar* L.), particularly at elevated water temperatures typical of Tasmanian summer conditions.

The maintenance requirement is influenced by many variables including species, sex, age, salinity, photoperiod, activity, body mass and temperature: body mass and temperature are the two most important factors for fish (Brett and Groves, 1979; Jobling, 1994; Lupatsch et al., 2001). Estimates of maintenance requirement have usually been calculated from unfed fish using one of two major techniques, either indirect calorimetry or comparative slaughter (Hepher, 1988; Lupatsch et al., 2001). Indirect calorimetry determines energy expenditure during a period without feed by measuring respiration (Brafield, 1985) whereas comparative slaughter techniques

determine energy and protein loss during starvation by calculating the difference between whole body composition before and after starvation (Lupatsch et al., 2001). Although indirect calorimetry has been widely used, comparative slaughter techniques are increasingly used for several reasons: the method is assumed to be straightforward; the method allows fish to be kept in groups and in tanks as normal; tanks allow fish to move freely and for a sufficiently long duration to lose measurable weight; greater accuracy and precision are required for respirometry (Azevedo et al., 1998; Lupatsch et al., 2001; Azevedo et al., 2004). Maintenance requirements are also predicted from relationships between nutrient intake and nutrient retention, this requires much larger experiments with replicated groups fed a range of rations (see Chapters 2 and 3). This task becomes very large when other variables such as water temperature or fish size have to be considered.

The first component of this study (Experiment 4.1) determined the maintenance energy and protein requirement at 16°C. The second component (Experiment 4.2) determined maintenance requirements at two temperatures, one set below (12°C) and one above (>18°C) a nominally “normal” summer temperature of 15-16°C (Miller et al., 2006). Data were then related to our previously determined relationships between protein and energy intake and growth (Chapter 2). This allowed evaluation of the method to provide “anchor” points, that differed with temperature, to adjust established ration-growth curves for different temperatures.

4.2. Material and methods

The experiments were conducted at the School of Aquaculture, University of Tasmania using 2000 L Rathbun tanks. The fish were held in a partial seawater recirculation systems and each tank was connected to a separate heat–chill unit. Water was treated through physical, UV and bio-filters with a replacement of approximately 60% week⁻¹. Water quality parameters (salinity, dissolved oxygen, pH, ammonia, and nitrite) were monitored at least twice weekly to ensure water quality remained well within limits recommended for Atlantic salmon in seawater (Tarazona and Munoz, 1995).

4.2.1. Experiment 4.1

Forty-nine all female Atlantic salmon (1139 ± 325 g, mean \pm sd) were used in the experiment. Twenty five fish were anaesthetised (50 mg L⁻¹ benzocaine), wet weight and fork length measured and each fish was then uniquely marked using a Panjet to inject alcian blue (2% solution in distilled water) dots into the ventral surface, with marks being placed between the pectoral and anal fins on either side of the body (Hart and Pitcher, 1969). Fish were then placed into a seawater recirculation system (see above) and held at $16.0 \pm 1.0^\circ\text{C}$. A further 24 fish were euthanised by anaesthetic overdose (100 mg L⁻¹ benzocaine), wet weight and fork length measured prior to them being frozen for whole body chemical analysis as the time-zero group (see below). Twenty four time-zero fish were matched by wet weight with the experimental fish to test whether pairing fish would provide more accurate data on changes in whole body chemical composition rather than using mean chemical composition values from the time-zero group and initial wet weight. Following 14 days without feeding, fish were euthanised by anaesthetic overdose, after which wet weight and fork length were measured. Fish were then frozen for whole body chemical analysis.

4.2.2. Experiment 4.2

One hundred and sixty Atlantic salmon were divided between 4 tanks so that duplicate tanks could be treated at low (12°C) and high (18°C) temperatures. Acclimation to the experimental temperatures commenced 22 days before the start of the experiment. The pre-experimental water temperature was 15.5 ± 0.5 °C, and was increased or decreased to 18°C and 12°C, respectively, by about 0.5°C daily. Water temperature was measured on a daily basis to monitor changes until it stabilized at 12°C or 18°C. During acclimation fish were hand fed twice per day to appetite on a commercial salmon feed (Skretting, Cambridge, TAS, Australia).

At the start of the experiment a time-zero group was sampled from the 4 tanks, 7 fish from each tank were killed following anaesthesia and frozen at -20°C used for initial whole body chemical composition (see below). Three further fish from each tank were then removed, killed, fork length and weight measured, dissected and the liver and viscera weighed. Prior to dissection, approximately 2-3 g white muscle tissue was quickly removed, frozen in liquid nitrogen and stored at -80°C for amino acid analysis (see Chapter 5).

The remaining 30 fish were anaesthetised (50 mg L⁻¹ benzocaine) and individually marked using alcian blue dye applied using a Panjet (Hart and Pitcher, 1969). The fork length and wet weight of individual fish were measured and the fish starved for 21 days. At the end of this time the fork length and wet weight of individual fish were measured. Fourteen fish from each tank were killed, 8 were used for an assessment of final whole body chemical composition and 6 for measuring the liver and viscera weights and white muscle tissue samples (see above).

4.2.3. Chemical analyses

Standard methods were used to determine whole body chemical composition (Carter and Hauler, 2000). Dry matter was measured by freeze drying to constant weight followed by 16 h drying at 105°C, crude protein by Kjeldahl ($N \times 6.25$), total lipid by Soxhlet and gross energy by bomb calorimeter (Gallenkamp Autobomb, calibrated with benzoic acid).

4.2.4. Statistical analysis

Unless stated results are given as mean \pm SEM (standard error of the means) using data collected from individually tagged individuals. Growth analyses are based on either tank means or individuals, the principle aim was to consider individual responses. Student t-test or one-way ANOVA was used to compare means, where there were differences a Tukey HSD multiple comparison was used. Linear regression was used to describe relationships between individuals. Significance was accepted at probabilities of 5% or less.

4.3. Results

4.3.1. Experiment 4.1

Over 14 days the mean wet weight of the maintenance group decreased from 1150 to 1110 g with a mean SGR of $-0.26 \pm 0.16 \text{ \% d}^{-1}$, condition decreased from $1.25 (\pm 0.11)$ to $1.17 (\pm 0.10)$. In comparison to the time-zero group the maintenance group of (unfed) salmon had significantly higher DM, crude protein and gross energy content (Table 4.1). This meant that it was not possible to calculate realistic (negative) changes in whole body chemical composition from the mean initial and individual final chemical composition values.

The data were re-calculated based on using the final whole body chemical composition with the change in wet weight of individual fish. This calculation provided values for the loss of dry matter ($-13.05 \pm 9.05 \text{ g DM}$; $-2.513 \pm 1.482 \text{ mg DM g}^{-1} \text{ d}^{-1}$), crude protein ($-6.05 \pm 4.03 \text{ g CP}$; $-0.377 \pm 0.232 \text{ mg CP g}^{-1} \text{ d}^{-1}$) and gross energy ($352 \pm 265 \text{ kJ}$; $-0.022 \pm 0.015 \text{ kJ g}^{-1} \text{ d}^{-1}$).

4.3.2. Experiment 4.2

Following a period of temperature acclimation, in which the fish were fed, and immediately prior to withholding feed (“maintenance requirement experiment”) there was no significant difference in initial wet weight between the two experimental temperatures ($F_{1,2} = 4.75$, $P = 0.161$) (Table 4.2). There was a significant difference in fork length ($F_{1,2} = 24.41$, $P = 0.039$) and therefore in condition ($F_{1,2} = 121.00$, $P = 0.008$) (Table 4.2), fish held at 12°C had a higher fork length and lower condition to those held at 18°C (Table 4.2). After 21 days wet weight final and condition had decreased at both temperatures, there was no significant difference in wet weight or SGR between the temperature treatments and the difference in fork length and condition at day zero remained (Table 4.2).

The changes in fish used for dissection and whole body chemical composition were analysed in more detail. Fork length, but not wet weight or condition, of the time-zero dissected group at 12°C was significantly higher than those at 18°C (Table 4.3). There were no significant differences in whole body chemical composition between the two temperatures (Table 4.3). After 21 days there was no significant difference between wet weight final or condition final, fish were longer at 12°C and the significant difference between initial fork length remained after 21 days (Table 4.3).

Initial analysis of the change in whole body chemical composition was based on mean values for the time-zero and starved groups at the two temperatures. The decreases in dry matter, both in absolute terms and as daily loss per gram of fish, were significantly higher at 18°C than at 12°C (Table 4.4). Probably due to the low CP and kJ values at time-zero this calculation resulted in crude protein and gross energy increasing for the 12°C fish. The data were recalculated using the final whole body chemical composition with the change in wet weight of individual fish to provide values for the change in dry matter, crude protein and gross energy loss (Table 4.4). For these data there were no significant differences in protein or energy loss between the temperatures.

4.3.3. Maintenance requirements

Weight exponents were used to calculate protein and energy loss in a form where comparisons could be made between fish with different wet weights. Exponents of -0.7 for protein and -0.8 for energy were used (Chapter 2). Protein losses were -0.498 (± 0.120), -0.490 (± 0.359) and -0.518 (± 0.110) g P kg^{-0.7} d⁻¹, respectively, for the temperatures 12, 16 and 18°C. Energy losses were -26.04 (± 6.46), -29.22 (± 23.96) and -26.90 (± 5.61) kJ kg^{-0.8} d⁻¹, respectively, for the three temperatures (Fig. 4.1).

4.3.4. Whole body chemical composition and condition

There was a relatively high level of individual variation in protein and energy loss, particularly in experiment 4.1 (Fig. 4.1). Consequently, the influence of initial condition on protein and energy loss was investigated. There were no significant correlations between initial condition and the loss of protein or energy at any temperature.

4.4. Discussion

The large size of the fish and short length of the experiments meant that there was only a small loss of wet weight and SGR values were -0.31, -0.26 and -0.35 % d⁻¹ for 12, 16 and 18°C treatments, respectively. In addition, there were few differences in whole body chemical composition between initial and final groups and between temperature treatments. In contrast, individual variation in whole body chemical composition was relatively high. This was especially the case for the 16°C treatment where the variation in wet weight was intentionally large (0.5 to 1.8 kg). These factors meant that the standard method, calculation of whole body protein and energy loss using the known initial wet weight in combination with the average whole body chemical composition of the zero-time group to calculate the initial protein and energy content, under-estimated the initial composition and even showed impossible increases in carcass energy content. For this reason the data were reanalysed, the loss of protein and energy was calculated from the change in wet weight and the final whole body chemical composition. This assumed the metabolism of protein and non-protein components occurred in the same proportions as found in the whole body after the period of starvation. In the initial stages of starvation lipid energy stores are used in preference to protein the calculation would have underestimated energy loss by a greater amount than protein loss. Consequently, the maintenance protein estimates from the current experiment were closer to estimates from other experiments than the energy estimates (Table 4.5).

After weight correction the mean values for protein and energy maintenance from losses at the three temperatures were quite similar and approximately 0.49 to 0.52 g protein kg^{-0.7} d⁻¹ and 26 to 29 kJ kg^{-0.8} d⁻¹. Fish in general and including juvenile salmon are well adapted to undergo periods without food, minimise energy expenditure and quickly recover growth on re-feeding. The similarity in losses between the three temperatures probably indicates that temperatures from 12 to 18°C are within the broad optimum range for salmon over which they have behavioural and physiological responses to reduce energy and protein losses. A comparative slaughter experiment was associated with the earlier feeding trial (Chapter 2) at 19°C and the data suggest that at 19°C salmon lose the ability to manage temperature and energy loss is

correspondingly large compared with the (low) values from the current experiment but also with other experiments conducted at near optimum temperatures (Table 4.5).

Chapter 2 provided salmon maintenance requirements that were calculated from relationships between nutrient intake and wet weight growth. Values of $4.6 \text{ g protein kg}^{-0.8} \text{ d}^{-1}$ and $155 \text{ kJ kg}^{-0.8} \text{ d}^{-1}$ were obviously higher than the values calculated from starving fish. Clearly, the metabolism of whole body protein and energy to sustain periods without food is a very different process to maintenance of feeding and growing fish. There will be a difference between the metabolism of starving and feeding fish irrespective of temperature. However, as noted above the difference is probably accentuated outside of the optimum range, this is discussed further in relation to barramundi (Katersky and Carter, 2007). Information on the effect of temperature on energy budgets and dietary aspects of protein and energy metabolism is limited (Medale and Guillaume, 2001). As discussed in the introduction, metabolism increases exponentially with temperature however this is not necessarily reflected by a smooth exponential increase in maintenance requirements. For example, maintenance requirements for rainbow trout increased by a greater amount between 10 and 15°C than between 15 and 20°C (Table 4.5). The maintenance requirements determined in the present study from intake-retention relationships appear high compared with the other species determined in the same way (Table 4.5).

Despite problems with calculation of changes in whole body composition there was a suggestion of an important threshold in the way salmon are able to manage maintenance energy expenditure between 18 and 19°C. This should be explored in further detail. Whilst using changes in whole body composition to determine quantitative estimates of requirements at different temperatures is not recommended the use of starvation experiments that incorporate physiological, biochemical and molecular approaches, as used in Chapter 6, would provide useful information about changes in physiology around temperature thresholds.

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Table 4.1

Basic data for maintenance requirement experiment 4.1 conducted on Atlantic salmon held at 16°C for 14 days

	Time-zero	Maintenance	P
Wet weight initial (g)	1128 ± 324	1150 ± 332	
Fork length initial (cm)	44.6 ± 3.3	45.2 ± 4.0	
Condition K initial	1.24 ± 0.15	1.25 ± 0.11	
Wet weight final (g)		1110 ± 323	
Fork length final (cm)		45.2 ± 4.0	
Condition K final		1.17 ± 0.10	
SGR (% d ⁻¹)		-0.259 ± 0.159	
Dry matter (% WW)	29.34 ± 4.05	31.83 ± 1.99	0.009
Crude protein (% WW)	13.89 ± 1.83	14.96 ± 1.04	0.015
Total lipid (% WW)	12.46 ± 2.97	13.72 ± 2.93	ns
Gross energy (kJ g WW ⁻¹)	7.81 ± 1.32	8.51 ± 1.11	0.049

Mean ± sd (n = 24, time-zero group; n = 25, maintenance group)

Table 4.2

Basic data for maintenance requirement experiment 4.2 conducted on Atlantic salmon held at 12°C and 18°C for 21 days

	12°C	18°C	P
Wet weight initial (g)	802 ± 11	778 ± 0	ns
Fork length initial (cm)	39.7 ± 0.2	38.7 ± 0.1	0.039
Condition K initial	1.28 ± 0.01	1.33 ± 0.00	0.008
Wet weight final (g)	752 ± 9	723 ± 3	ns
Fork length final (cm)	40.4 ± 0.3	38.9 ± 0.1	0.029
Condition K final	1.13 ± 0.01	1.22 ± 0.01	0.024
SGR (% d ⁻¹)	-0.309 ± 0.009	-0.345 ± 0.017	ns

Mean ± SEM (n = 2, 40 individually marked fish in 2 tanks per temperature)

Table 4.3

Whole body chemical composition and organ somatic indices for maintenance requirement experiment 4.2 conducted on Atlantic salmon held at 12°C and 18°C for 21 days

	12°C	18°C	P
<i>Time-zero group</i>			
Wet weight initial (g)	739 ± 20	720 ± 12	ns
Fork length initial (cm)	38.8 ± 0.1	38.2 ± 0.1	0.045
Condition K initial	1.23 ± 0.05	1.27 ± 0.04	ns
Dry matter (% WW)	31.44 ± 1.13	34.83 ± 1.31	ns
Crude protein (% WW)	15.53 ± 0.18	17.33 ± 0.60	ns
Gross energy (kJ g WW ⁻¹)	8.33 ± 0.32	9.33 ± 0.37	ns
<i>Starvation group</i>			
Wet weight initial (g)	795 ± 15	753 ± 18	ns
Fork length initial (cm)	40.1 ± 0.1	39.1 ± 0.2	0.033
Condition K initial	1.23 ± 0.03	1.26 ± 0.05	ns
Wet weight final (g)	745 ± 10	702 ± 20	ns
Fork length final (cm)	40.1 ± 0.1	39.1 ± 0.2	0.033
Condition K final	1.15 ± 0.02	1.17 ± 0.05	ns
Dry matter (% WW)	32.71 ± 0.18	33.74 ± 0.35	ns
Crude protein (% WW)	16.87 ± 0.05	17.46 ± 0.28	ns
Total lipid (% WW)	16.42 ± 0.63	17.85 ± 0.19	ns
Gross energy (kJ g WW ⁻¹)	9.03 ± 0.01	9.32 ± 0.04	0.022
Mean ± SEM (n = 2, 7 individually marked fish from 2 tanks per temperature)			

Table 4.4

Change in whole body chemical composition for maintenance requirement experiment 4.2 conducted on Atlantic salmon held at 12°C and 18°C for 21 days

	12°C	18°C	P
Dry matter change (g)	-5.69 ± 2.14	-26.18 ± 2.53	0.025
Dry matter change ² (g)	-16.22 ± 1.35	-18.07 ± 0.16	ns
Dry matter change ² (mg g ⁻¹ d ⁻¹)	-0.981 ± 0.064	-1.144 ± 0.039	ns
Crude protein change (g)	2.15 ± 0.96	-8.20 ± 2.24	0.051
Crude protein change ² (g)	-8.38 ± 0.71	-9.37 ± 0.16	ns
Crude protein change ² (mg g ⁻¹ d ⁻¹)	-0.508 ± 0.035	-0.593 ± 0.024	ns
Gross energy change (kJ)	131.0 ± 20.0	-472.0 ± 9.0	0.001
Gross energy change ² (kJ)	-448 ± 39	-498 ± 4	ns
Gross energy change ² (kJ g ⁻¹ d ⁻¹)	-0.027 ± 0.002	-0.032 ± 0.001	ns

Mean ± SEM (n = 2, 7 individually marked fish from 2 tanks per temperature)

² Calculated from wet weight loss and final whole body chemical composition

Table 4.5
Maintenance requirements for energy and protein of fish species fed formulated feeds

Species	Method Details	Temperature / weight	Energy ^a (kJ kg ^{-0.8} d ⁻¹)	Protein ^a (g P kg ^{-0.7} d ⁻¹)	Source
Sea bream	R-G	21-24°C/50g	43 ^b	0.86 ^c	Lupatsch et al., 2001
Rainbow trout		7.5°C	12		*Cho & Slinger, 1980
		10°C	19		*
		15°C	35		*
		20°C	38		*
		8°C/100g	29		*
		18°C/100g	40-48		*
Atlantic salmon	R-G	19°C/450g	155	4.58	Chapter 2
			185 ^b	2.19 ^c	Chapter 2
	CS	19°C/450g	124	0.82	Chapter 2
	CS	12°C/750g	26	0.50	Chapter 4
	CS	16°C/1110g	29	0.49	Chapter 4
	CS	18°C/700g	27	0.52	Chapter 4

R-G, ration growth curve; CS, comparative slaughter

^a Maintenance for wet weight unless stated otherwise, weight exponents may differ between studies but all data were weight corrected

^b Maintenance for whole body energy

^c Maintenance for whole body protein

* cited from (Medale and Guillaume, 2001)

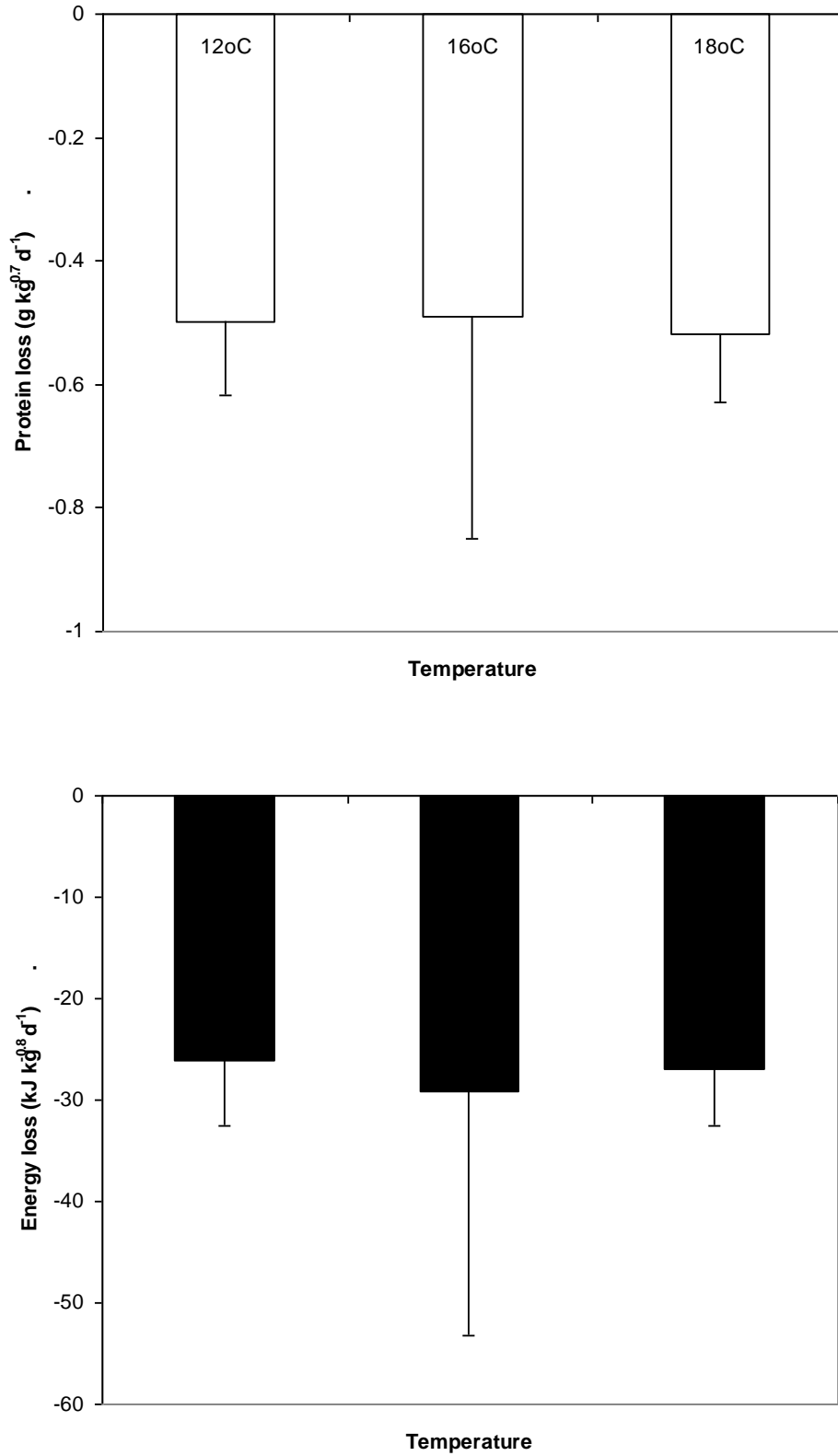


Fig. 4.1. The loss of a) protein ($\text{g P kg}^{-0.7} \text{d}^{-1}$) and b) energy ($\text{kJ kg}^{-0.8} \text{d}^{-1}$) at 12, 16 and 18°C by seawater Atlantic salmon of 780 to 1150 g.

Chapter 5

Assessment of possible temperature dependent limiting nutrients in seawater Atlantic salmon, *Salmo salar* L., at two temperatures**Abstract**

The aim of this experiment was to test whether the white muscle free pool amino acid (AA) concentration would differ between temperatures of 12 and 18°C and whether changes in white muscle free pool AA concentration could be used to indicate a limiting amino acid in 0.7 kg seawater Atlantic salmon (*Salmo salar* L.). Two methods were used, a ninhydrin-based method measured concentrations of total free pool ninhydrin positive substances (NPS) and HPLC measured free AA individually. White muscle tissue was used because the greatest proportion of available AA are used in biosynthesis and retained in new tissue, therefore the balance of indispensable amino acids (IAA) is likely to be most critical in this tissue. Atlantic salmon were fed to satiation and acclimated to 12 and 18°C, following which they were starved for 21 days. The starved fish at 12°C had a significantly higher free pool NPS concentration than at 18°C. The concentration was $58.74 \pm 2.60 \mu\text{mol NPS g}^{-1}$ wet tissue, which was $15.24 \mu\text{mol NPS g}^{-1}$ higher than at 18°C. Starvation, but not temperature, had a significant effect on AA concentrations. Lysine, threonine and proline concentrations were lower and asparagine higher after starvation. There were no differences between the summed concentration of AA, IAA or NIAA. However, the IAA had a lower concentration relative to NIAA (IAA/NIAA) and AA (IAA/AA) after starvation. Starvation resulted in significantly higher expression of muscle cathepsin L and proteasome β subunit N3 at 12°C. Overall, the experiment found no evidence of major differences in amino acid concentration due to temperature. Differences were due only to starvation. However, there were differences between temperature in the expression of two components of protein degradation. It appeared that at 12°C the fish had lower condition, had up-regulated muscle protein breakdown to recycle amino acids in the muscle free pool, presumably to act as an energy source. An additional observation was that the data from the initial group suggested that, in normally feeding Atlantic salmon, the dietary amino acids were not limiting at either temperature.

5.1. Introduction

Knowledge of amino acid metabolism in relation to temperature may provide information about the availability of nutrients not only for growth but also for maintenance. Under conditions where non-protein energy is limiting, amino acids will be used as the major energy source. The use of amino acids as energy substrates may in turn lead to imbalances in the amino acids remaining (Carter et al., 2000; Carter and Houlihan, 2001). For example, diets containing ingredients with lower energy availability caused a greater overnight energy deficiency that resulted in significant changes in amino acid free pool concentrations (Gomez-Requeni *et al.*, 2003; Gomez-Requeni *et al.*, 2004). It has also been demonstrated that in Atlantic salmon fed low rations specific indispensable amino acids (IAA) have lower white muscle free pool concentrations in relation to other IAA (Carter et al., 2000). This suggests deficient IAA would then limit protein synthesis and growth (Carter et al., 2000; Carter and Houlihan, 2001). Elsewhere in this report it is hypothesised that elevated temperatures lead to increased use of amino acids as energy substrates (Chapter 9). The aim of this experiment was to test in Atlantic salmon (*Salmo salar* L.) whether the white muscle

free pool amino acid concentration would differ at two temperatures and whether changes in amino acids could be used to indicate a limiting amino acid.

The experiment used seawater Atlantic salmon that were not fed for 21 days and were sampled at the end of the experiment described in Chapter 4. This provided Atlantic salmon at 12 and 18°C. Two methods were compared, one measured total free pool amino acid concentration using a ninhydrin-based method (Shumway et al., 1977) and the other measured amino acids individually (Carter et al., 1995). White muscle tissue was used because the proportion of available amino acids used in the synthesis of new tissue is greatest in white muscle, therefore the balance of IAA is likely to be most critical. At the start of the experiment the initial group sampled was representative of salmon fed to satiation at 12 and 18°C and data used to compare normally feeding fish.

It should be noted that the analysis in Chapters 2 and 3 showed that at 19°C Atlantic salmon performed according to responses expected from general understanding of nutritional physiology at normal temperatures. Dietary induced differences in feed intake appeared to be the main determinant of differences in growth performance. The factorial approach achieved the required aims for Chapters 2 and 3 but there was no indication any nutrient limitation. A nutrient limitation at elevated temperature had been hypothesised when developing the grant and the design of the proposed experiment was changed to investigate whether a focus on free pool amino acid concentrations would be more sensitive and reveal any temperature effects.

5.2. Material and methods

5.2.1. Fish and facilities

The experiment was conducted at the School of Aquaculture, University of Tasmania. Atlantic salmon, *Salmo salar* L., (180 g mean weight) were obtained from the Huon Aquaculture Company (Huonville, Tasmania, Australia) and stocked into 2000-L Rathbun tanks at 50 fish per tank. Each tank was connected to a heat – chill unit and temperature maintained at $15.5 \pm 0.5^\circ\text{C}$ with a 12:12 photoperiod. The fish were held in a partial seawater recirculation system. Water was treated through physical, UV and biofilters with a replacement of approximately 60% week⁻¹. The fish were fed twice a day on a commercial salmon feed (Skretting Industries Ltd, Cambridge, Tasmania, Australia). Water quality parameters (salinity, DO, pH, ammonia, nitrate, nitrite) were monitored twice a week to ensure water quality remained well within limits recommended for Atlantic salmon (Tarazona and Munoz, 1995). One hundred and sixty fish were divided between 4 tanks with duplicate tanks for low (12°C) and high (18°C) temperatures. Acclimation to the experimental temperatures was done over 22 days, the initial water temperature was $15.5 \pm 0.5^\circ\text{C}$ and changed by about 0.5°C daily after which the fish were left at the final temperatures for a further 2 weeks. Fish were fed twice per day to appetite.

5.2.2. Experiment

At the start of the experiment, seven fish from each tank were killed following anaesthesia (50 mg L⁻¹ benzocaine) and frozen at -20°C used for an assessment of initial chemical composition (see Chapter 4). A further 3 fish from each tank were

killed, weighed, the liver and viscera dissected and weighed, and approximately 2-3 g white muscle tissue removed, frozen in liquid nitrogen and stored at -80°C for amino acid analysis (see below).

The remaining 30 fish were anaesthetised (50 mg L^{-1} benzocaine) and individually marked using Panjet with alcian blue dye (Hart and Pitcher, 1969). The fork length and wet weight of individual fish were measured and the fish were not fed for 21 days. After this time fork length and wet weight of individual fish were measured. Fourteen fish from each tank were killed, 8 were used for an assessment of final whole body chemical composition (Chapter 4). A further 6 fish from each tank were killed, weighed, the liver and viscera dissected and weighed, and approximately 2-3 g white muscle tissue removed, frozen in liquid nitrogen and stored at -80°C for amino acid analysis (see below). Samples of white muscle were dissected from 3 fish per tank and used for analysis of gene expression (Chapter 1).

5.2.3. Free pool amino acid concentration

Ninhydrin-positive substances (NPS) were measured according to (Shumway et al., 1977). Approximately 100 mg frozen muscle tissue was weighed and homogenized in 6 ml 80% ethanol. After centrifugation (10 min, 4°C , 5500 g) 50 μl of supernatant were removed and 950 μl of distilled water added to 4 ml of ninhydrin solution and 2 ml of hydrazine sulphate solution. Once the ninhydrin solution has been added the tubes were kept dark, heated at 100°C for 10 min, cooled in air and then cold water. Leucine standards were made up in the range $0 - 600\text{ }\mu\text{g ml}^{-1}$ ($0 - 4.58\text{ }\mu\text{M NPS ml}^{-1}$) in 80% ethanol. The optical density was determined at 570 nm and results were expressed as $\mu\text{M NPS g}^{-1}$ wet tissue.

Approximately 200 mg of white muscle tissue was weighed into a tared glass test tube and homogenized in 4 ml of absolute ethanol plus 0.1 ml norvaline ($10\text{ }\mu\text{mol ml}^{-1}$) as an internal standard, and 0.9 ml distilled water. The homogenate was centrifuged (10 min, 4°C , 6000 g) and a 50 μl sub-sample of supernatant transferred to a microcentrifuge tube and freeze dried. Analysis was exactly according to previous methods (Lyndon et al., 1993; Carter et al., 1995; Carter et al., 2000). The freeze dried samples were sent to Australian Protein Analysis Facility (APAF, Macquarie University, NSW, Australia). In the present experiment, tryptophan and arginine were not reported because the tryptophan concentration was too low and arginine was co-eluting with a contaminant peak found in the blank.

5.2.4. Statistical analysis

Unless stated results are given as mean \pm SEM (standard error of the means) using data collected from individually tagged individuals. Growth analyses are based on either tank means or individuals, the principle aim was to consider individual responses. Student t-test or one-way ANOVA was used to compare means, where there were differences a Tukey HSD multiple comparison was used. Linear regression was used to describe relationships between individuals. Significance was accepted at probabilities of 5% or less.

5.3. Results

5.3.1. Free pool amino acid concentrations

The white muscle free pool concentration measured by NPS was $58.74 \pm 2.60 \mu\text{mol NPS g}^{-1}$ wet tissue, which was $15.24 \mu\text{mol NPS g}^{-1}$ wet tissue or nearly a third higher than at 18°C . Furthermore, without feeding the free pool concentration increased at both temperatures. At 12°C the increase was $10.42 \mu\text{mol NPS g}^{-1}$ wet tissue whereas it was $4.12 \mu\text{mol NPS g}^{-1}$ wet tissue at 18°C (Fig. 5.1).

Two-way ANOVA of temperature and time showed that temperature did not have a significant effect but time had a significant effect, but this was restricted to only a few AA and measures. There was no significant interaction between time and temperature. Of the IAA both lysine and threonine concentrations were significantly lower after starvation. For NIAA proline was also significantly lower but ASX was significantly higher after starvation. There were no differences between the summed concentration of AA, IAA or NIAA. However, the IAA had a lower concentration relative to NIAA (IAA/NIAA) and AA (IAA/AA) after 21 days of starvation.

5.3.2. Gene expression

Gene expression after 21 days of starvation was significantly higher at 12°C for cathepsin L (Fig. 5.3) and for the proteasome β subunit N3 (Fig. 5.4) but not changed in the other 4 genes investigated (Fig. 5.2 – 5.4).

5.4. Discussion

This experiment investigated the white muscle free pool amino acid concentrations to determine whether there were differences in total, total indispensable (IAA), total non-indispensable (NIAA) or individual amino acids at low and high temperatures. The purpose was to explore the use of an alternative approach to identify possible limiting amino acids (Carter and Houlihan, 2001; Gomez-Requeni et al., 2003; Gomez-Requeni et al., 2004). Numerous experiments show that white muscle free pool amino acid concentrations are responsive to both major and subtle changes in the nutritional status of fish (Mommsen et al., 1980; Anderson et al., 1993; Arzel et al., 1995). However, different amino acids respond differently to a nutritional factor. For example, only some amino acids show a break-point response in the muscle free pool at the dietary requirement for that amino acid (Arzel et al., 1995). The strategy was adopted because although the analysis of growth in relation to digestible protein and digestible energy intake did not suggest a nutrient limitation at 18°C (Chapter 2) the intake-growth approach may not be sensitive enough to reveal small or impending deficiencies. Additionally, our research on barramundi suggested free pool amino acid concentrations were decreased at elevated temperature.

Ninhydrin-positive substances (NPS) were used as a relatively straightforward method for measuring the total free pool amino acid concentration in the white muscle. There was no significance difference due to temperature at the start of the experiment. These fish had been at their respective temperatures for 14 days and fed to satiation, as such they represented the metabolic situation in feeding fish at each temperature. Based on NPS, the total free pool concentrations of feeding fish were not affected by

elevated temperature and this suggested Atlantic salmon utilise amino acids in similar ways at 18°C and 12°C. After 21 days starvation at 12°C the fish had a significantly higher free pool NPS concentration than at 18°C. This suggested white muscle protein was being used more extensively as an energy substrate at the lower temperature. In sockeye salmon increased protein degradation resulted in higher levels of NPS, such as small peptides, in the cell free pools (Mommsen et al., 1980).

There were no significant differences between the two temperatures in the muscle free pool amino acid concentrations. The few differences in AA concentrations were due to starvation (not temperature). The concentrations of the IAA lysine and threonine and of the NIAA proline decreased whereas asparagine + aspartate (ASX) increased. The changes in lysine (x0.5), proline (x0.28) and ASX (x4) were relatively large. Whilst there were no differences between the summed concentrations of total AA, IAA or NIAA the proportion of IAA in relation to both NIAA and total AA decreased significantly. The use of body reserves for energy during starvation depends on the initial energy reserves and involves a complex sequence of mobilising substrates. In salmon fat reserves, particularly around the viscera are used at the start of starvation. The muscle of salmonids accounts for about 50% of wet weight and acts as a major energy reserve. It is likely that sometime after starvation starts the muscle free pool AA concentration will increase due to the onset of muscle breakdown and then decrease as metabolism and protein degradation slow during more advanced stages of starvation. For example, wild sockeye salmon undergoing their spawning migration do not feed and therefore must use muscle as an energy source. After completing part of their migration (600 km) the free pool concentration of histidine decreased dramatically but for many AA it increased, lysine and arginine increased by about x7 (Mommsen et al., 1980). In seawater rainbow trout a month of starvation provided an interesting comparison with the present experiment, whilst in trout the total free pool AA concentration was significantly higher unlike the salmon in which it was lower (Kaushik and Luquet, 1977). The proportion of IAA decreased in both fish species and was likely to be partly due to rapid removal from the free pool and recycling into proteins via protein synthesis (Anderson et al., 1993; Arzel et al., 1995; Carter et al., 2000). In this case the large decrease in the concentrations of lysine and threonine suggest these are likely to be the most limiting AA, although there was not a significant temperature effect the possible lysine deficiency is further emphasised by the lysine concentration at 12°C being less than 60% the 18°C value.

It is likely that the salmon at 12°C were in a more advanced state of starvation than the 18°C fish. Although counter intuitive it was due to their lower condition which was significantly lower at the start and, therefore, also at the end of the starvation period. Their final condition of 1.09 is relatively low for 700 g Atlantic salmon (Austreng et al., 1987). Furthermore, whole body energy content was significantly lower and lipid and protein numerically lower than at 18°C (Chapter 4). Evidence for greater reliance on muscle protein metabolism in the 12°C salmon is provided by the increased free pool NPS concentration, generally lower muscle free pool AA concentrations and the up-regulation of two genes involved in protein degradation. Expression of cathepsin L and proteasome β subunit N3 was significantly higher at 12°C. In contrast the two calpains measured did not appear to respond to temperature. This indicates up-regulation of two of the three proteolytic systems, the ubiquitin-proteasome complex and the lysosomal system that includes several cathepsins (Mommsen, 2004). Cathepsin D has broad specificity and its biochemical activity was significantly higher

as starvation progressed in the migrating sockeye salmon (Mommsen et al., 1980; Mommsen, 2004). Cathepsin D is viewed as being of more universal importance in protein degradation with cathepsin L sometimes being involved (Mommsen, 2004). In contrast, our data show differences in cathepsin L expression and only suggest cathepsin D is up-regulated in the 12°C salmon. Calpains may also have a role during starvation and Mommsen (2004) suggested a role in the breakdown of muscle Z-discs. In the present experiment the expression of calpain 2 is almost identical between the two temperatures but there is a suggestion of up-regulation of calpain 1 expression. In the current experiment the subtle differences and lack of significance are indicative of two important effects related to differences in the position of fish along a starvation pathway: as a group the 12°C fish are further advanced in the use of muscle protein, and individual variation is likely to be considerable.

The concentrations reported in the present study were generally typical of values measured for other salmonids (Espe et al., 1993; Carter et al., 1995; Carter et al., 2000; Espe et al., 2007). The NPS method estimated a higher final total free amino acid concentration than the sum of individual amino acid concentrations measured by HPLC. The main reason relates to the lack of specificity of the NPS method, ninhydrin positive substances include low molecular weight peptides and molecules with a free α -amino group (as well as not including molecules such as proline where there is no free α -amino group). Small differences were also due to arginine and tryptophan not being reported. In relation to tryptophan it should be noted that the preparation of free pool samples did not include an acid hydrolysis phase (used to break down protein) so the low level was not due to the method. There are few biological studies that compare the two methods directly and the preferred method will depend on experimental aims and constraints. Although analysing individual AA is time consuming and more costly it is more informative since analysis of individual AA will reveal subtle but important differences.

Starvation was not the principle focus of the research and used only as a way to investigate amino acid metabolism and to examine whether it would highlight an amino acid deficiency due to temperature. Although there was no temperature effect, the results support the combined use of gene expression with physiological measures to highlight subtle changes in nutritional status of Atlantic salmon. Overall the current experiment provided one of the few studies available on changes in gene expression related to different measures of nutritional status in Atlantic salmon. Salmon at 12°C had lower condition and were at a more advanced stage of starvation, they were relying more on muscle protein as an energy source and this was reflected by differences in free pool amino acid concentrations and changes in gene expression related to specific protein degradation pathways.

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Table 5.1
Basic data for Atlantic salmon at 12 and 18°C before and after 21 days starvation.

	12°C	18°C	P
<i>Time-zero group</i>			
Wet weight initial (g)	882 ± 39	805 ± 50	ns
Fork length initial (cm)	41.0 ± 0.6	39.0 ± 1.5	ns
Condition K initial	1.28 ± 0.05	1.36 ± 0.08	ns
<i>Starvation group</i>			
Wet weight initial (g)	694 ± 61	738 ± 57	ns
Fork length initial (cm)	38.3 ± 1.1	38.1 ± 1.0	ns
Condition K initial	1.21 ± 0.02	1.31 ± 0.02	0.004
Wet weight final (g)	654 ± 59	693 ± 54	ns
Fork length final (cm)	38.8 ± 1.1	38.2 ± 1.0	ns
Condition K final	1.09 ± 0.02	1.22 ± 0.02	<0.001
SGR (% d ⁻¹)	-0.290 ± 0.026	-0.303 ± 0.080	ns

Mean ± SEM (Time-zero from 3 individually marked fish from each of 2 tanks per temperature, n = 6. Final from 6 individually marked fish from each of 2 tanks per temperature, n = 12)

Table 5.2

Individual free pool amino acid concentrations ($\mu\text{mol g}^{-1}$ wet muscle) in white muscle from Atlantic salmon at 12°C and 18°C in Atlantic salmon before and after 21 days of starvation

Amino acid	Initial 12°C	Initial 18°C	Final 12°C	Final 18°C	P
Histidine	0.41 ± 0.06	0.71 ± 0.46	0.53 ± 0.20	0.54 ± 0.30	<i>ns</i>
Isoleucine	0.20 ± 0.11	0.17 ± 0.07	0.26 ± 0.12	0.40 ± 0.29	<i>ns</i>
Leucine	0.46 ± 0.24	0.37 ± 0.14	0.39 ± 0.18	0.58 ± 0.41	<i>ns</i>
Lysine	0.53 ± 0.33	0.43 ± 0.11	0.19 ± 0.11	0.33 ± 0.19	<i>time</i>
	0.48 ± 0.09		0.26 ± 0.04		<i>0.013</i>
Methionine	0.18 ± 0.09	0.12 ± 0.02	0.14 ± 0.05	0.19 ± 0.13	<i>ns</i>
Phenylalanine + Tyrosine	0.37 ± 0.17	0.26 ± 0.03	0.21 ± 0.11	0.30 ± 0.24	<i>ns</i>
Threonine	1.79 ± 0.89	1.44 ± 0.39	0.82 ± 0.35	1.00 ± 0.68	<i>time</i>
	1.61 ± 0.26		0.90 ± 0.11		<i>0.013</i>
Valine	0.49 ± 0.27	0.43 ± 0.14	0.48 ± 0.24	0.77 ± 0.59	<i>ns</i>
Alanine	2.44 ± 0.87	1.30 ± 0.27	3.27 ± 1.48	3.01 ± 2.16	<i>ns</i>
Glycine	4.91 ± 1.85	4.25 ± 0.83	3.11 ± 1.87	4.12 ± 2.93	<i>ns</i>
Proline	0.60 ± 0.44	0.68 ± 0.10	0.16 ± 0.06	0.20 ± 0.16	<i>time</i>
	0.64 ± 0.12		0.18 ± 0.03		<i><0.001</i>
Serine	0.81 ± 0.34	0.38 ± 0.11	0.72 ± 0.34	0.92 ± 0.59	<i>ns</i>
Asparagine + Aspartate	0.20 ± 0.13	0.12 ± 0.07	0.56 ± 0.28	0.77 ± 0.48	<i>time</i>
	0.16 ± 0.04		0.64 ± 0.08		<i>0.007</i>
Glutamine + Glutamate	1.30 ± 0.43	1.46 ± 0.17	1.57 ± 0.77	2.50 ± 1.15	<i>ns</i>
∑IAA	4.43 ± 2.10	3.93 ± 0.47	3.02 ± 1.24	4.00 ± 2.77	<i>ns</i>
∑NIAA	10.26 ± 4.12	8.18 ± 1.19	9.37 ± 4.44	11.40 ± 7.26	<i>ns</i>
∑AA	14.69 ± 6.20	12.11 ± 1.66	12.39 ± 5.54	15.40 ± 9.95	<i>ns</i>
∑IAA/∑NIAA	0.43 ± 0.04	0.48 ± 0.01	0.34 ± 0.06	0.35 ± 0.10	<i>time</i>
	0.45 ± 0.02		0.34 ± 0.02		<i>0.005</i>
∑IAA/∑AA	0.30 ± 0.02	0.33 ± 0.01	0.25 ± 0.04	0.25 ± 0.05	<i>time</i>
	0.31 ± 0.01		0.25 ± 0.01		<i>0.006</i>

IAA, indispensable amino acids; NIAA, non-indispensable amino acids

Mean ± SEM (n=6 initial, n=12 final). Two-way ANOVA between time and temperature, significant differences noted (Interaction between time and temperature was not significant).

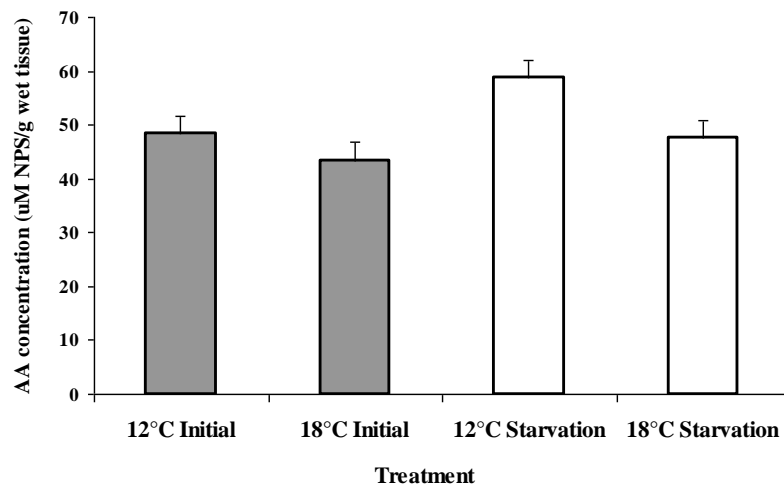


Fig. 5.1. Total free pool amino acid concentration ($\mu\text{M NPS g}^{-1}$ wet tissue) in white muscle from Atlantic salmon at 12 and 18°C before (initial) and after 21 days starvation.

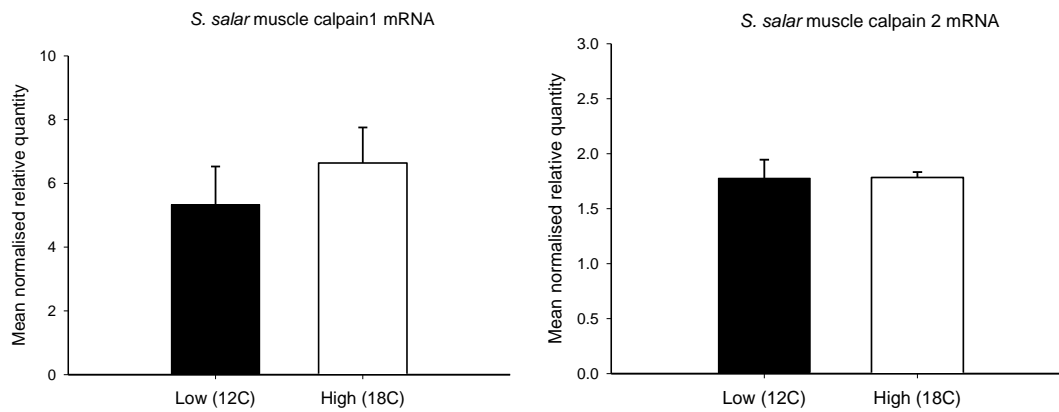


Fig. 5.2. Expression of calpain 1 and calpain 2 in white muscle from Atlantic salmon at 12 and 18°C after 21 days starvation.

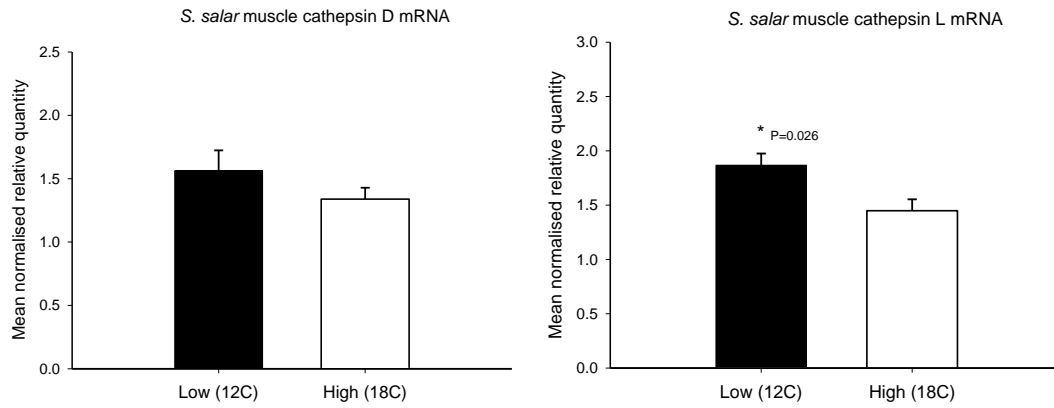


Fig. 5.3. Expression of cathepsin D and L in white muscle from Atlantic salmon at 12 and 18°C after 21 days starvation.

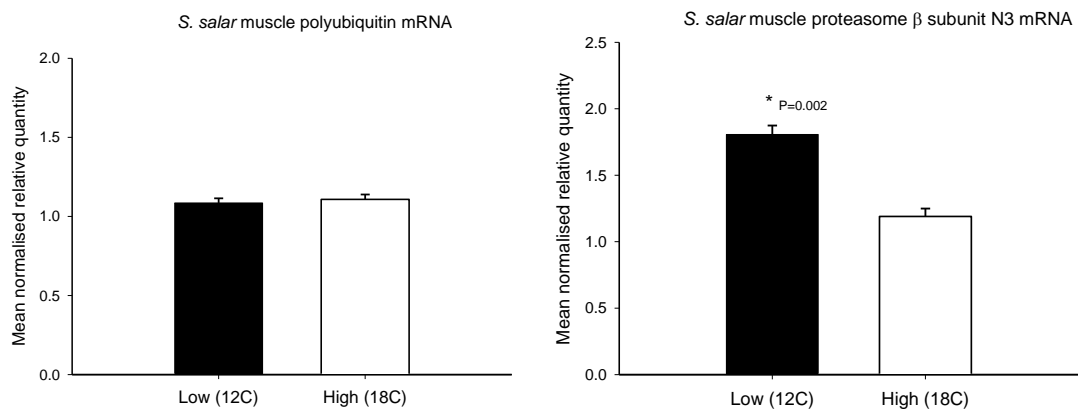


Fig. 5.4. Expression of polyubiquitin and proteasome β subunit N3 in white muscle from Atlantic salmon at 12 and 18°C after 21 days starvation.

Chapter 6

Assessment of growth performance and protein turnover of individual seawater Atlantic salmon, *Salmo salar* L. at an elevated temperature of 19°C**Abstract**

The experiment aimed to investigate protein turnover in Atlantic salmon through analysis of nitrogenous excretion and changes in gene expression of marker genes for protein degradation pathways. Four diets were formulated to be either high (H) or low (L) in protein and energy: L:vL (low protein and very low energy); L:L (low protein and low energy); H:L (high protein and low energy) and H:H (high protein and high energy). Salmon were fed to satiation until they had approximately doubled in weight from approximately 200 to 500 g. Although there was a significant difference in growth amongst the diets, L:vL produced significantly higher growth than H:L, this was explained by the low feed intake on H:L. At the end of the growth component daily rates of nitrogenous excretion were measured in individual fish and related to diet and to that days feed and protein-nitrogen intake. Individual differences in feed intake had a greater effect on nitrogenous excretion than did diet composition. There were no differences amongst diets in the linear relationships between nitrogen intake and excretion, consequently all data were combined. There were significant positive relationships between crude protein nitrogen intake and ammonia, urea and total nitrogen excretion. Nitrogen excretion as a percentage of nitrogen intake was, on average, 42.0%, with no significant difference between the diets. Urea-nitrogen excretion as a percentage of ammonia-nitrogen excretion was 19.8% on average, with no significant difference between the diets. There were no significant differences in gene expression amongst the dietary treatments.

6.1. Introduction

A variety of experimental approaches are available for understanding the relationships between feeds, feeding, nutrition and physiology of fish and how they impact on growth performance: clearly the most appropriate method depends on the experimental aim (Jobling, 1985; Carter et al., 2001; Glencross et al., 2007). An additional concern is how to interpret experimental data to have on-farm application. One approach is to develop sampling procedures that can be used on-farm to provide clear information about the fish. This is arguably a straightforward task when data on the average weight of fish or diagnosis of a disease is required. An example of when the task is more complex is when the aim of sampling is to reveal detailed information about the impact of a factor on a long term process such as growth rate by considering a short term nutritional response. There has been a long history of attempts to develop the use of various biochemical measures as quick assessment tools, a good example is the use of growth correlates such as RNA:DNA ratios that have been used many times in fisheries to provide an estimate of recent growth (Houlihan et al., 1993). The rapid development of biotechnology has the potential to provide a number of different types of “tool” to assess and to predict performance, one approach is to use a limited number of specific genes for which changes in their expression can be interpreted through a fundamental and empirical understanding of the underlying physiological processes.

An experiment was conducted to establish methods for assessing protein turnover in Atlantic salmon (*Salmo salar* L.). Three of the four diets with varying digestible protein to digestible energy ratios used previously (Chapters 2 and 3) were used in this

experiment. A new diet with a lower energy content was also included (L:vL). The methods that were tested to measure protein turnover were the measurement of nitrogenous excretion via ammonia and urea, and the measurement of changes in expression of key genes involved in protein degradation (Chapter 1). An attempt to measure protein synthesis using a labelled ^{15}N -protein did not work due to samples not containing sufficient ^{15}N for measurement, this was despite a small initial experiment that confirmed ^{15}N could be measured in the end-products. In any case, the method is only applicable to laboratory situations and facilities for stable isotope analysis are limited in Australia. Consequently, the combination of gene expression with standard measures of growth provide the most practical combination of assessment methods that have potential for development as on-farm measures of fish nutritional status. This experiment aimed to examine whether changes in fish performance and protein-nitrogen metabolism would be reflected by differences in gene expression. To be applicable, on-farm data should be considered at the level of an average group response.

6.2. Materials and methods

6.2.1. Experimental diets

Four experimental diets were produced on a cold press and coated with fish oil by mixing pellets with oil. The diets were formulated to be either high (H) or low (L) in both digestible protein and digestible energy, resulting in four different DP:DE ratios (Table 6.1): L:L low protein low energy; L:H low protein high energy; H:L high protein low energy and H:H high protein high energy. The protein was supplied from anchovy meal (Austral Group, Peru), soybean meal (Cargill, VIC, Australia), corn gluten meal (Hunter Grain, NSW, Australia), feather meal (Camelleri Stockfeeds, NSW, Australia) and whole wheat (Cooperative Bulk Handling, Australia). Oil was supplied from Peruvian anchovy (Sindicato Pesquero, Peru). A vitamin/mineral premix was supplied as a commercial preparation according to known requirements for salmonids. Feeds were stored at $1 - 2^{\circ}\text{C}$ for the duration of the experiment. To introduce a stable isotope label a portion of each feed was ground to powder and a ^{15}N -protein added. However, as noted above the experiment was unsuccessful and is not discussed further.

6.2.2. Growth experiment

The experiment was conducted at the School of Aquaculture, University of Tasmania. Atlantic salmon (*Salmo salar* L.) smolt were obtained from Wayatinah Salmon Hatchery (SALTAS, TAS, Australia), evenly divided between twenty-four 300-l tanks in a partial recirculation system (Carter and Hauler, 2000) and acclimated to saltwater (30 ppt) over 6 days in increments of 5 ppt. The temperature was then steadily raised from 12°C to 19°C over 12 days. During acclimation fish were fed the H:H diet. Water was treated through physical and bio-filters with partial water changes as required to ensure water quality parameters (DO, pH, ammonia, nitrite and nitrate) stayed within the limits recommended for Atlantic salmon (Tarazona and Munoz, 1995).

At the start of the experiment, fish were anaesthetised (50 mg l^{-1} , benzocaine) and wet weight and fork length measured. Four individually marked fish were randomly

allocated to one of twenty-four 300-l tanks. Initial whole body chemical composition of the fish was calculated using data from previous experiments on similar sized fish (Chapter 2). Fish were fed to satiation at 0900 and 1530 every day and feed intake determined by direct observation. At the end of each excretion experiment (see below), the fish were not fed the evening meal, and were euthanised with an overdose of benzocaine the next morning. Final wet weight and fork length were determined, and the fish were stored at -20°C. Samples of white muscle were also taken for analysis of gene expression (see Chapter 1).

6.2.3. Nitrogenous excretion

Each excretion experiment lasted four days, with one row of tanks used each time. Prior to the commencement of each experiment, the two smallest fish were culled from each tank. The larger 2 fish were hand fed with the ¹⁵N diet and feed intake of each determined by observing the number of pellets eaten. The fish which ate the least was culled, leaving one fish per tank. The fish were then fed as normal and on the normal unlabelled diet between 0630 and 0800 on the following morning and evening. Water samples were taken from all tanks and stored at -20°C. Tanks were drained to the top of the cone base, leaving 117L. Tanks were then filled with fresh saltwater to 300L. The time between samples was approximately 11 h, with 1 h for feeding fish and flushing tanks.

The total ammonia present in the water samples was determined using the salicylate-hypochlorite assay (Bower and Holm-Hansen, 1980). Urea concentration was determined by treating samples with urease followed by the salicylate-hypochlorite assay (Bower and Holm-Hansen, 1980; Carter and Brafield, 1991).

6.2.4. Chemical analysis

Standard methods were used to determine dry matter (freeze dried to constant weight); nitrogen (Kjeldahl using a copper catalyst); total lipid (Bligh and Dyer, 1959); energy (bomb calorimeter: Gallenkamp Autobomb, calibrated with benzoic acid); ash (AOAC, 1995).

6.2.5. Statistical analysis

Data are reported mean \pm SEM (standard error of the means). Comparison between means from satiation treatments (n=3) was by one-way ANOVA, with post-hoc comparison by Tukey. The slopes of the regression lines were compared by ANCOVA; where there was no significant difference between treatments the data were pooled. Significance was accepted at probabilities of 5% or less. Analyses were carried out with SPSS for Windows.

6.3. Results

6.3.1. Growth and feed intake

There were significant differences amongst diets for final wet weight, weight gain and SGR (Table 6.2). For final weight and weight gain L:V was significantly greater than H:L. However, differences between initial weight meant that SGR was

significantly higher for L:vL compared to H:H, L:L and H:L were not different from these two diets. Feed intake of H:L was significantly lower than the other three diets and crude protein intake was significantly higher for L:vL than H:L.

There were no significant differences amongst diets in the regressions for protein gain against protein intake and data were combined (Fig. 6.1). The relationship predicted a maintenance protein intake of $3.9 \text{ g.kg}^{-0.7} \cdot \text{d}^{-1}$ and retention efficiency, above maintenance of 0.597 (60%).

6.3.2. Nitrogenous excretion

During the 3 day excretion trial, crude protein nitrogen intake was not significantly different amongst the diets (Table 6.3). Ammonia, urea and total nitrogen excretion were not significantly different amongst diets. Total nitrogen excretion as a percentage of nitrogen intake was, on average, 42.0%, with no significant difference between the diets. N-urea excretion as a percentage of N-NH₃ excretion was 19.8% on average, with no significant difference between the diets. There were positive relationships between crude protein nitrogen intake and the three measures of nitrogen excretion (Fig. 6.2). There was a negative relationship between crude protein nitrogen intake and the proportion of nitrogen excreted as urea (Fig. 6.3).

Nitrogen budgets were estimated using nitrogen intake, growth and excretion of fish in the present experiment. Apparent digestibility was estimated from values from Chapters 2 and 3 (Table 6.4). Assuming nitrogen intake was 100% the unknown or error component ranged between 3.0 and 4.8%. Retention accounted for the greatest part of the budget, then ammonia excretion and then faecal and urea losses, which were about the same.

6.3.3. Gene expression

There were no significant differences in the expression of four enzymes involved in protein turnover (Fig. 6.4).

6.4. Discussion

The experiment aimed to investigate protein turnover through construction of nitrogen budgets and investigation of nitrogen flux in Atlantic salmon at an elevated temperature. The four diets used previously (Chapters 2 and 3) with different digestible protein digestible energy content were compared. Nitrogen budgets provide a further level of detail about the metabolism of different diets and are particularly useful in providing information about nitrogenous excretion (Brett and Zala, 1975; Carter and Bransden, 2001).

In the present experiment the design involved using six replicate tanks initially containing four fish to produce one fish that was feeding and growing at a high rate and was therefore representative of the satiation fed fish held in groups. Atlantic salmon are a difficult species to conduct long-term physiology experiments with. They are easily stressed and difficult to keep alone. They also show strong agonistic behaviours and can form strong hierarchies when in small groups (Carter et al., 1994). It was for these reasons that the present experiment culled out fish from a group to

leave one fish on which to conduct the measurement of excretion. Consequently the data in Table 6.2 represent the performance of the groups of fish which contained fish of variable performance, most tanks having at least one fish which fed rarely if at all. Table 6.3 concerns the data generated from the single high performing fish and a critical aspect of these data is the equal feed intake. This allowed comparison of nitrogenous excretion across diets that was independent of feed intake. Apparent digestibility from the previous experiments was used. This was because the single fish used to measure nitrogenous excretion would not produce enough faeces nor could it be collected during the 3 day experiment. It was not possible to remove the fish for stripping. The experiment was large and used six replicates per diet to take account of greater variation between replicates, this used all 24 tanks in the system. All fish were individually identified so that wet weight growth could be calculated accurately, the change in whole body composition used the previous experiments to estimate initial composition. Feed intake and therefore nitrogenous excretion over the 3 day excretion experiment was assumed to reflect the situation over the whole of the growth trial. Individual identification also meant that the gene expression measurements could be related to long term growth and to excretion measurements.

Overall the trends amongst diets followed those expected from the previous experiments. There were no differences amongst diets in nitrogenous excretion or in expression of genes involved in protein turnover. Differences were explained by differences in feed intake which also resulted in differences in nitrogen intake. Analysis of nitrogenous excretion in relation to nitrogen intake did not show any diet effects and the combined data predicted strong linear relationships between intake and excretion (Beamish and Thomas, 1984; Carter and Brafield, 1992).

Urea excretion in salmonids is stated to be independent of nutrient intake (Brett and Zala, 1975) whereas it appears to be correlated in other fish taxa such as flatfish (Dosdat et al., 1996; Verbeeten et al., 1999). In the present experiment urea excretion was significantly related to nitrogen intake, this is likely to be explained by the relationships between nutrient intake and nutritional status of individual salmon and by the relationship between nutritional status and urea excretion. Higher feed intake over the 3 day experiment was likely to have reflected generally higher performance and higher nutritional status. A major component of urea excretion is due to breakdown of purines and reflects turnover of nucleic acids, this might be greater in fish of higher nutritional status and explain the relationship between nitrogen intake and urea excretion. The nitrogen budget showed urea was less than 10% of total nitrogenous excretion, this is typical of salmonids and clearly of less significance than in other fish taxa where it may reach 30% (Beamish and Thomas, 1984; Dosdat et al., 1996; Verbeeten et al., 1999).

Nitrogen retention (PN) in the budgets was around 45% and shows that the fish were performing well. Nitrogen retention calculated in a nitrogen budget includes endogenous and exogenous excretion. Endogenous excretion is comparable with maintenance excretion and analysis of the relationship between nitrogen intake and retention calculates retention, above maintenance, of 60%. The relationship also predicted a protein maintenance requirement of $3.9 \text{ g protein kg}^{-0.7} \text{ d}^{-1}$. The comparable value from $2.2\text{-}3.9 \text{ g protein kg}^{-0.7} \text{ d}^{-1}$, the difference probably highlights the problem of extrapolating too far “backwards” using data clustered around high nitrogen intakes.

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Table 6.1
Ingredient composition of feed kernal and chemical composition of experimental feeds¹

	Diet			
	L:vL	L:L	H:L	H:H
Ingredient composition (g kg ⁻¹)				
Fish meal	480	427	534	480
Corn gluten	60	53	67	60
Soybean meal	92	82	102	92
Feather meal	50	44	56	50
Wheat	130	150	111	130
Vit/Min premix	3	3	3	3
Fish oil	85	141	127	185
Bentonite	100	100	0	0
Chemical composition (g kg ⁻¹ DM)				
Dry matter (g kg ⁻¹)	940.08	898.38	901.52	897.52
Crude protein (g kg ⁻¹)	426.57	380.12	465.05	447.82
Total lipid (g kg ⁻¹)	214.14	285.08	305.76	312.05
Total ash (g kg ⁻¹)	143.61	135.27	91.07	86.49
Gross energy (MJ kg ⁻¹ DM)	19.91	21.47	22.72	23.33
CP:GE	21.43	17.71	20.47	19.19

¹ Feed kernel was coated with fish oil and final composition is shown under chemical composition.

Diets: L:vL (low protein: very low energy), L:L (low protein:low energy), H:L (high protein:low energy) and H:H (high protein:high energy).

Table 6.2
The performance of Atlantic salmon fed diets with different DP:DE at 19°C

Parameter	Unit	Diet				F	P
		L:vL	L:L	H:L	H:H		
Initial weight	(g)	226.67 ± 35.28	230.28 ± 36.21	158.97 ± 14.03	265.67 ± 44.61	1.68	0.204
Final weight	(g)	551.53 ^a ± 50.55	519.06 ^{ab} ± 56.08	347.9 ^b ± 23.06	535.81 ^{ab} ± 68.34	3.29	0.042
Weight gain	(g.d ⁻¹)	3.45 ^a ± 0.31	3.04 ^{ab} ± 0.27	1.98 ^b ± 0.20	2.84 ^{ab} ± 0.32	4.98	0.010
SGR	(% d ⁻¹)	0.96 ^a ± 0.03	0.88 ^{ab} ± 0.04	0.82 ^{ab} ± 0.05	0.76 ^b ± 0.04	4.19	0.019
Feed Intake	(g.d ⁻¹)	3.50 ^a ± 0.27	3.19 ^a ± 0.25	2.02 ^b ± 0.20	3.08 ^a ± 0.30	6.23	0.004
CPI	(g.d ⁻¹)	1.49 ^a ± 0.11	1.31 ^{ab} ± 0.10	0.94 ^b ± 0.09	1.36 ^{ab} ± 0.13	4.53	0.014

Mean (± SEM, n = 6). Different letters denote significant differences between means.

Diets: L:vL (low protein: very low energy), L:L (low protein:low energy), H:L (high protein:low energy) and H:H (high protein:high energy).

CPI, crude protein intake

Table 6.3
Nitrogenous excretion of Atlantic salmon fed diets with different DP:DE at 19°C

Parameter	Unit	Diet				F	P
		L:vL	L:L	H:L	H:H		
CP-NI	(mg N.kg ^{-0.7} .d ⁻¹)	1174.26 ±224.50	1165.12 ±171.86	990.08 ±146.23	994.62 ±91.77	1.13	0.359
Total N	(mg N.kg ^{-0.7} .d ⁻¹)	486.43 ±79.16	490.48 ±74.35	431.28 ±67.31	424.67 ±20.89	0.80	0.511
N-NH ₃	(mg N.kg ^{-0.7} .d ⁻¹)	397.93 ±28.26	401.66 ±41.41	332.28 ±48.00	338.64 ±34.58	0.31	0.820
N-Urea	(mg N.kg ^{-0.7} .d ⁻¹)	88.49 ±19.58	88.82 ±18.60	85.73 ±16.10	86.03 ±5.92	0.57	0.642
Total N % of CP-NI		41.27 ±0.93	42.10 ±0.85	41.81 ±0.81	42.80 ±0.94	1.91	0.161
N-Urea % of N-NH ₃		18.88 ±0.28	18.19 ±0.27	21.72 ±0.39	20.48 ±0.18	2.48	0.091

Mean (± SEM, n = 6). Different letters denote significant differences between means.

Diets: L:vL (low protein: very low energy), L:L (low protein:low energy), H:L (high protein:low energy) and H:H (high protein:high energy).

CP-NI, crude protein nitrogen intake

Table 6.4
Nitrogen budget for Atlantic salmon fed diets with different DP:DE at 19°C

	Unit	Diet			
		L:vL	L:L	H:L	H:H
C _N	(%)	100.00	100.00	100.00	100.00
P _N	(%)	45.34 ±1.16	43.66 ±1.43	43.16 ±0.56	45.00 ±1.54
F _N ¹	(%)	10.00	10.50	8.80	9.20
A _N	(%)	32.84 ±1.38	33.58 ±1.11	33.54 ±1.66	33.49 ±1.56
U _N	(%)	8.43 ±1.84	8.52 ±1.03	9.68 ±2.16	9.31 ±1.26
Unknown	(%)	3.38 ±0.69	3.74 ±0.44	4.82 ±1.03	3.00 ±0.52

¹ Apparent digestibility used values from Chapters 2 and 3.
Nitrogen intake (C_N), gain (P_N), faecal loss (F_N), ammonia (A_N) and urea (U_N) excretion.
Diets: L:vL (low protein: very low energy), L:L (low protein:low energy), H:L (high protein:low energy) and H:H (high protein:high energy).

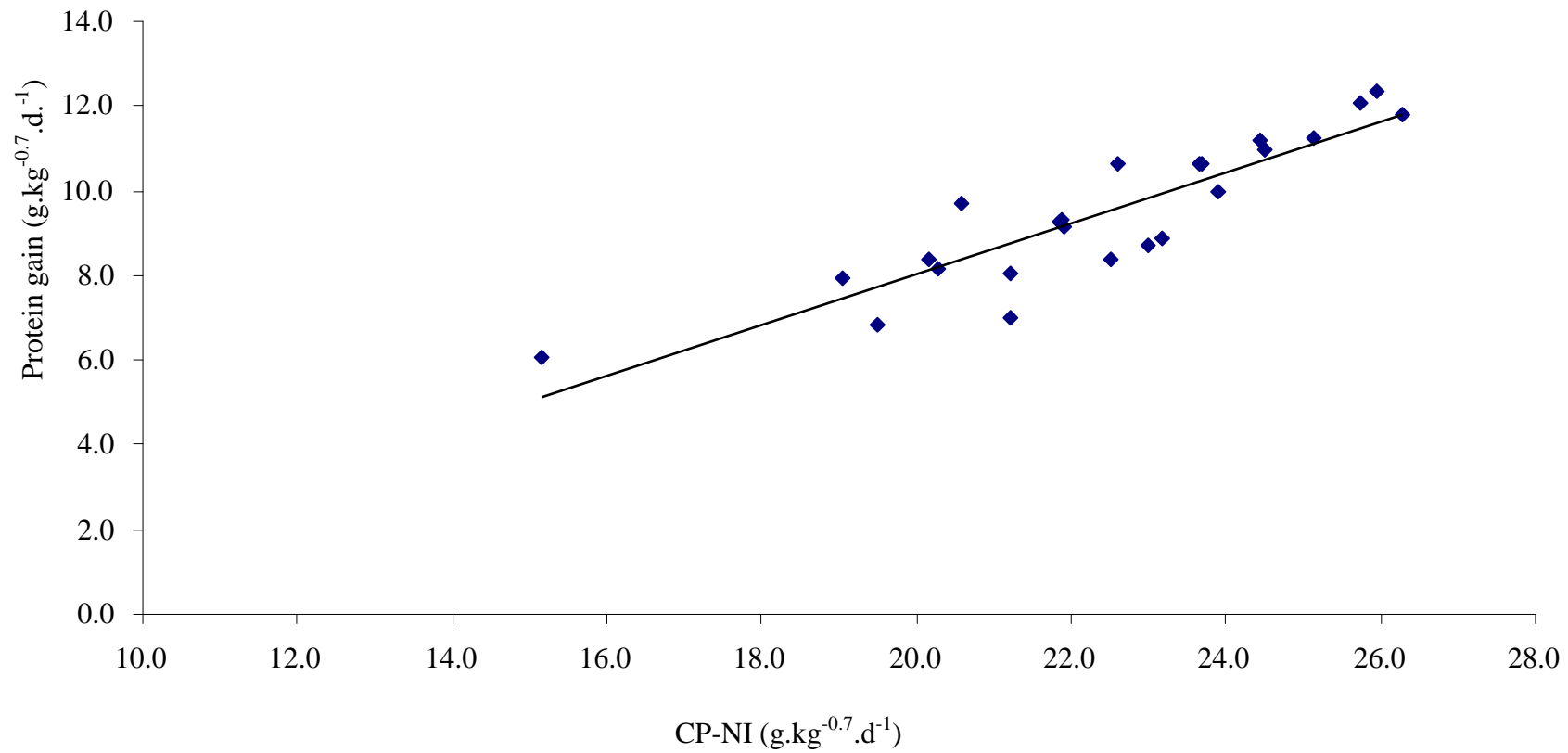


Fig. 6.1.
The relationship between CP-NI and protein gain and for Atlantic salmon at 19° fed diets with different CP:CE (Protein gain = 0.597(0.04).CP-NI + 3.90(0.06) ($R^2 = 0.80$, $F = 33.365$, $P = 0.000$, $n = 2$)).

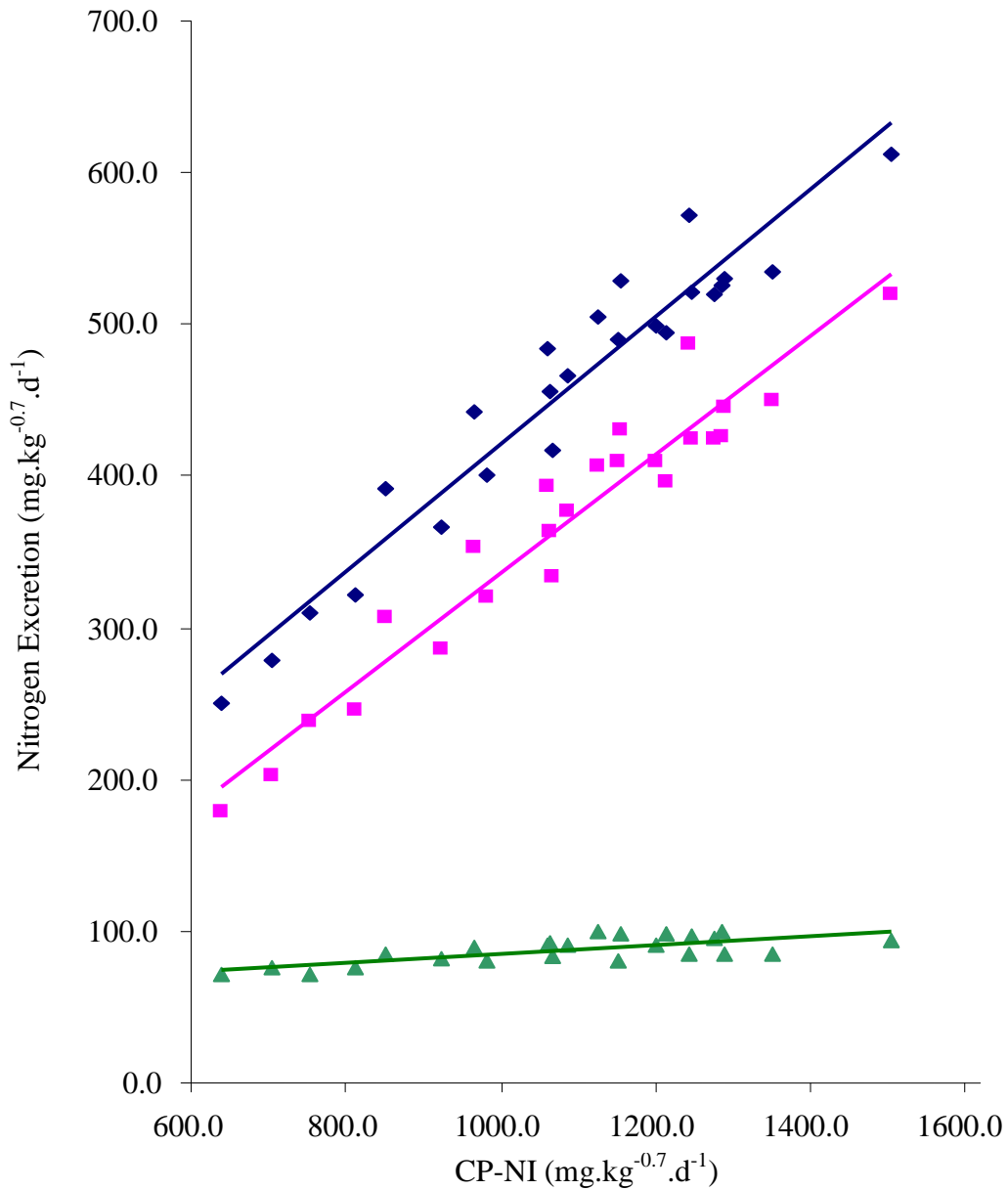


Fig. 6.2. The relationships between crude protein nitrogen intake (CP-NI) and nitrogen excretion (total, ammonia and urea) of Atlantic salmon at 19°C fed diets with different DP:DE (◆ Total nitrogen excretion; ■ Ammonia-N excretion; ▲ Urea-N excretion): $N\text{-Total} = 0.419 (\pm 0.02) \cdot \text{CP-NI} + 2.30 (\pm 2.62)$ ($R^2 = 0.93$, $F = 554.4$, $P = 0.000$, $n = 24$); $N\text{-NH}_3 = 0.391 (\pm 0.04) \cdot \text{CP-NI} - 54.54 (\pm 9.99)$ ($R^2 = 0.92$, $F = 10.7$, $P = 0.003$, $n = 24$); $N\text{-Urea} = 0.048 (\pm 0.01) \cdot \text{CP-NI} + 56.83 (\pm 8.25)$ ($R^2 = 0.51$, $F = 423.7$, $P = 0.000$, $n = 24$).

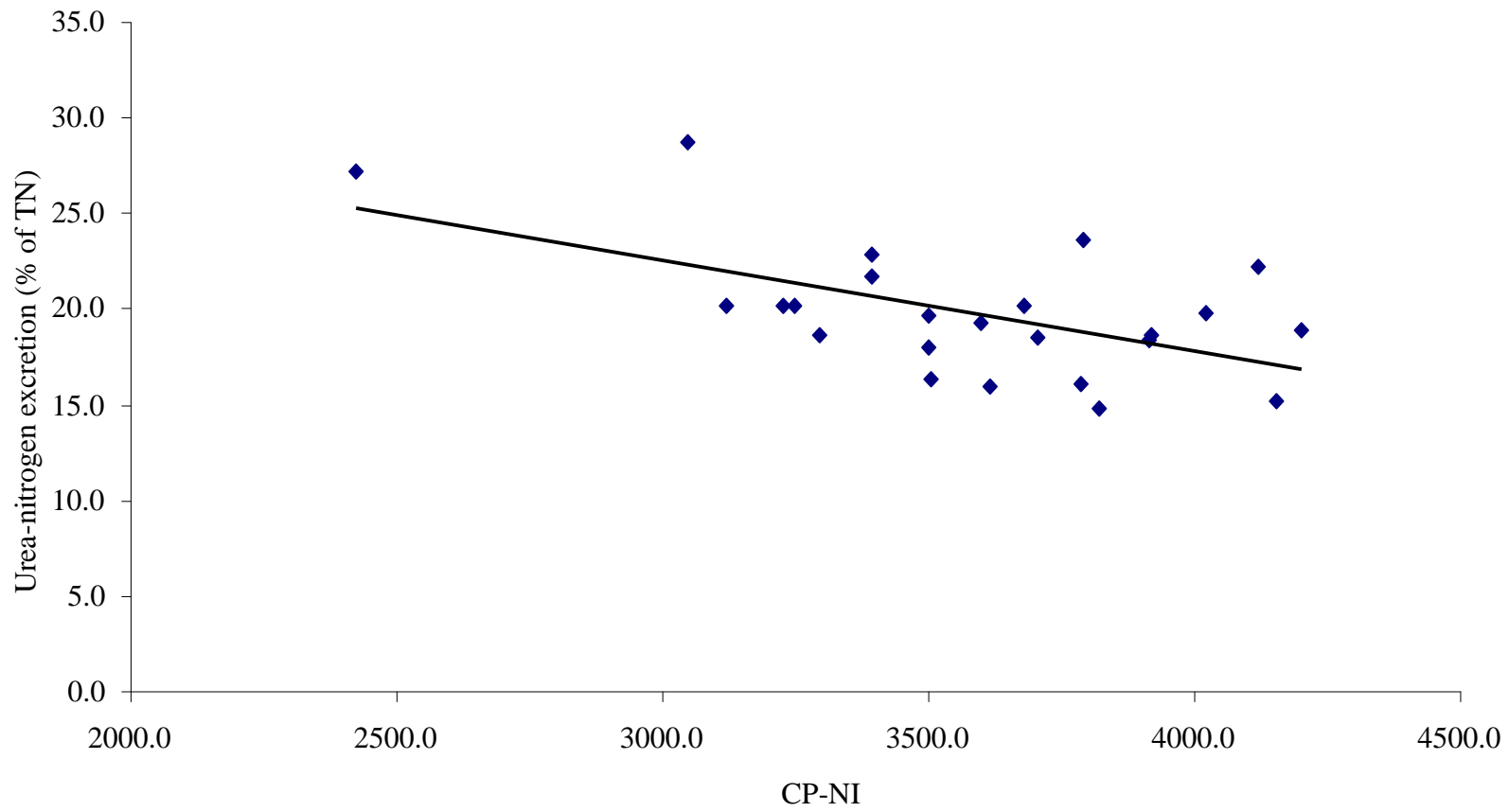


Fig. 6.3.
The relationship between urea as a percent of nitrogen excretion and nitrogen intake (Urea-nitrogen (%TN) = -0.0047x + 36.645 (R² = 0.32, n = 24)).

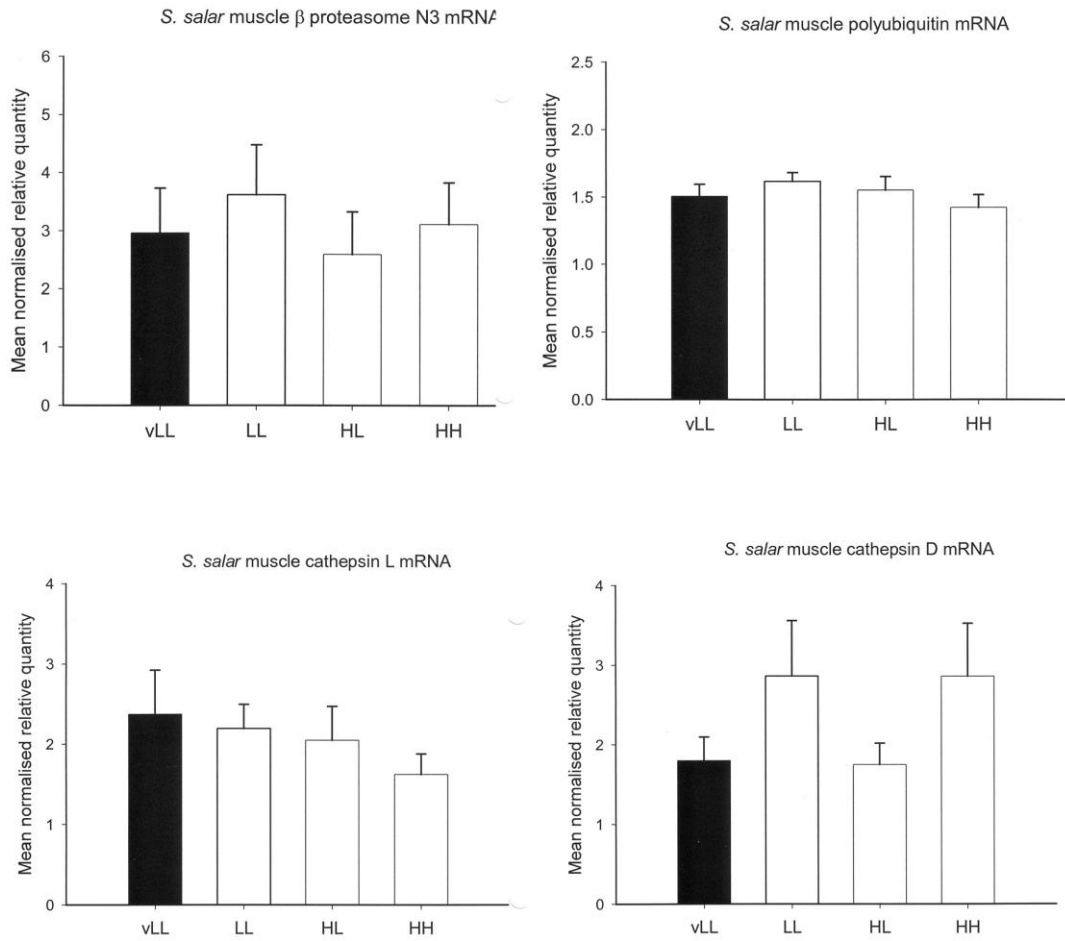


Fig. 6.4. Expression of a) β proteasome sub-unit N3, b) polyubiquitin, c) Cathepsin L, and d) Cathepsin D from Atlantic salmon fed diets of different DP:DE content and compared to L:vL diet. Diets: L:vL (low protein: very low energy), L:L (low protein:low energy), H:L (high protein:low energy) and H:H (high protein:high energy).

Chapter 7*

**The effect of temperature on growth performance in barramundi,
Lates calcarifer (Bloch)**

*Adapted from: Katersky, R.S., Carter, C.G. 2007. High growth efficiency occurs over a wide temperature range for juvenile barramundi *Lates calcarifer* fed a balanced diet. *Aquaculture* 272: 444-450.

Abstract

Temperature has a marked and direct effect on many of the key physiological processes in ectotherms, including fish. Results from two growth trials on juvenile barramundi (~3-5 g) were used to model the effects of temperature on the feed intake and growth performance, measured as specific growth rate (SGR), productive protein value (PPV) and productive energy value (PEV), across temperatures 21-39 °C (at 3 °C intervals). There were no significant differences between the chemical composition of the fish reared at different temperatures. Optimal temperatures were determined from quadratic polynomials: maximum feed intake ($\text{g}\cdot\text{d}^{-1}$), maximum growth (SGR, $\%\cdot\text{d}^{-1}$) and growth efficiency (PPV and PEV, %) occurred at 32.8, 31.4, 31.2 and 30.2°C, respectively. These models also determined that maximum growth occurred approximately 4°C higher than previously demonstrated. Feed intake, SGR and growth efficiency remained $\geq 90\%$ of the maximum biological response over an 8°C temperature range. These models suggest that juvenile barramundi are eurythermal and therefore maximise growth by adopting strategies to optimise nutrient utilization over as wide a temperature range as possible. Furthermore, the decrease in growth efficiency at extreme (high) temperature occurs over only a few degrees and highlights the upper thermal tolerance of barramundi.

7.1. Introduction

Temperature has a marked and direct effect on many of the key physiological processes in ectotherms including fish (Brett and Groves, 1979) on which there have been numerous studies (Jobling, 1981; McCarthy et al., 1998; Jonassen et al., 1999) and reviews (Elliott, 1982; Jobling, 1997). Each species has a range of temperature over which it survives, the thermal tolerance range, and a narrower range where growth occurs. As temperature increases within the thermal tolerance range feed intake increases to a maximum and then decreases rapidly prior to the upper thermal tolerance limit (Jobling, 1994), while simultaneously the metabolic rate increases exponentially. Consequently, at temperatures which approach the upper thermal tolerance limit, the metabolic demands will increasingly account for a larger proportion of the ingested energy until growth is compromised (Brett and Groves, 1979; Jobling, 1994). The optimal temperature for growth reflects the temperature where the difference between the ingested energy and the energy expenditures is largest and energy partitioned into growth is maximal. Nutrient retention efficiency reflects how efficiently animals make use of nutrients, particularly when fed one formulated diet (Carter and Houlihan, 2001).

Barramundi (*Lates calcarifer*) provides an excellent model species to examine the effects of temperature on feed intake and growth efficiency because it has a wide thermal tolerance range (15-40°C), in addition to being a globally important fisheries and aquaculture species (Carter et al., In press). Barramundi culture has expanded to geographic locations outside its natural range and therefore culture is often conducted

at extreme temperatures which approach the lower and upper thermal tolerance limits of the species (Katersky and Carter, 2005). Interestingly, this situation is comparable to that of Atlantic salmon (*Salmo salar*) which have a temperature tolerance of -0.5-25°C (Wallace, 1993) and are farmed under widely different temperature regimes (Jobling et al., In press). It is common in the salmon industry for fish to be exposed to low temperatures, <5 °C, in the northern hemisphere (Koskela et al., 1997) and high temperatures 20-22°C in the southern hemisphere (Roberts et al., 2001). Models for feed intake, growth and growth efficiency over the thermal tolerance range for wild salmon indicate that growth efficiency is maintained at a high level over a wide temperature range (Forseth et al., 2001). Growth efficiency in barramundi is also maintained at high levels over a wide temperature range (27-36 °C, Katersky and Carter, 2005).

The aim of the current paper is to present models for feed intake, growth and growth efficiency, expressed in relation to protein and energy retention, for juvenile barramundi at temperatures which span the thermal tolerance range for the species (21-39°C). Two experiments were conducted. Experiment 1 examined temperatures from 21 to 33 °C (in 3°C increments, Katersky and Carter, 2007) and experiment 2 examined temperatures 27, 33, 36 and 39 °C (Katersky and Carter, 2005). By combining these experiments it was possible to determine optimal temperatures and temperature ranges for maximum feed intake, growth and growth efficiency. Such information has potential uses for farm management as well as establishing baseline data for a species prior to nutritional assessments.

7.2. Materials and methods

7.2.1. Experimental diet

A standard diet was formulated according to known dietary requirements for barramundi (Boonyaratpalin and Williams, 2002). The diet was formulated to contain 50% crude protein (CP), 19.7 MJ kg⁻¹ gross energy (GE) and as 24.5 g CP MJ GE⁻¹ (Katersky and Carter, 2005, Table 7.1). Fish meal and fish oil were supplied by Skretting (Tasmania, Australia). Vitamins and minerals were supplied by Sigma-Aldrich Pty. Ltd (Sydney, Australia), Vitamin C was supplied as Stay-C from Roche Pharmaceuticals (Roche Vitamins Australia Ltd., Sydney, Australia). Diets were pelleted through a cold pellet press (CL-2 laboratory pellet mill, California Pellet Mill Co., San Francisco, CA, USA)

7.2.2. Growth experiments

Methods for the growth experiments have been previously described (Katersky and Carter, 2005; 2007). Briefly, stock juvenile barramundi (1 g) were obtained from WBA Hatcheries (South Australia, Australia). Fish were maintained at the University of Tasmania under constant environmental conditions (salinity: 10‰; photoperiod: 24 h light; temperature: 27°C) in 150-L aquaria. During the acclimation period, fish were fed to satiation twice daily and temperatures were adjusted 1°C d⁻¹ towards their experimental temperature (experiment 1: 21, 24, 27, 30 or 33°C, experiment 2: 27, 33, 36 or 39°C). At the start of each experiment, 60 fish from each temperature treatment were anesthetized (100 mg L⁻¹, benzocaine) and individual weights (g) were measured (Table 7.2) an additional 10 fish were randomly sampled for initial whole-body

chemical composition. Fish were randomly separated into 3 19-L carboys. Water quality was monitored 3 times week⁻¹ and water changes were done as necessary to keep water quality within the limits for barramundi (Tucker et al., 2002). The experiments were conducted in identical recirculating systems each consisting of 3 19-L carboys with a trickle biofilter on each system (Katersky and Carter, 2005). Each system was held at a constant temperature and was monitored daily. The standard diet was fed to all fish at all times to satiation twice daily at 0900 and 1800 during the experimental period (experiment 1: 22 d, experiment 2: 20 d). A pre-weighed ration was provided to each tank and if completely consumed additional pellets were counted out and provided until feeding ceased. Any uneaten pellets are siphoned out after 10 min and counted in order to determine total daily feed intake. The following equations were used to calculate specific growth rate (SGR), productive protein value (PPV) and productive energy value (PEV): $SGR (\% \cdot d^{-1}) = 100((\ln(W_2/W_1)/d)$; $PPV (\%) = (\text{fish protein gain (g CP)} \cdot \text{total protein consumed (g CP)}^{-1}) \cdot 100$; $PEV (\%) = (\text{fish energy gain (MJ} \cdot \text{kg}^{-1}) \cdot \text{total energy consumed (MJ} \cdot \text{kg}^{-1})^{-1}) \cdot 100$. At the end of the growth experiments, five fish were killed with an overdose of benzocaine (400 mg L⁻¹) from each replicate tank, autoclaved (Williams et al., 1995) and freeze-dried to a constant weight in order to determine whole-body chemical composition (Table 7.3).

7.2.3. Chemical analysis

Standard methods were used to determine dry weight (freeze drying to a constant weight); crude protein (Kjeldahl); total lipid (Bligh and Dyer, 1959); energy (bomb calorimeter); ash by combustion at 550 °C for 16 h (AOAC, 1995).

7.2.4. Statistical analysis

Data are presented as mean \pm SEM (standard error of the means). Relationships between body composition, feed intake, growth and growth efficiency and temperature were modelled using quadratic polynomials (SigmaPlot, version 8.0). Analysis of covariance was used to adjust for the initial size differences between experiments. Where the covariate was significant, adjusted means were used in the analysis for each relationship. The optimal temperature was determined by the maximum biological response, while the optimal temperature ranges were considered where the biological response was $\geq 90\%$ of the maximum response.

7.3. Results

Fish in experiment 1 had a four fold increase in body weight at temperatures above 27°C, while in experiment 2 this magnitude of increase in body weight was only seen at temperatures 33 and 36°C (Table 7.2). In both experiments, mean treatment survival was high, above 95% (Table 8.2). No significant relationships between temperature and whole-body chemical composition (crude protein ($r^2 = 0.266$; $F_{2,6} = 1.09$; $P = 0.395$), total lipid ($r^2 = 0.024$; $F_{2,6} = 0.074$; $P = 0.929$) and energy ($r^2 = 0.128$; $F_{2,6} = 0.44$; $P = 0.663$)) were found when the experiments were combined, however, when each experiment was examined individually some differences in the body composition occurred between the different experimental temperatures (Katersky and Carter, 2005; 2007, Table 7.3).

Significant relationships occurred between temperature and feed intake, growth and growth efficiency (Table 7.4). The model for temperature and feed intake determined that maximum feed intake occurred at 32.8°C and the range of temperature where feed intake remained $\geq 90\%$ of the maximum was 28.8-36.8°C (Fig. 7.1a). The model for temperature and SGR predicted a maximum SGR of 7 %·d⁻¹ at 31.4°C while the optimal range was determined to occur between 28-34.8°C (Fig. 7.1b). The model for temperature and PPV (Fig. 7.1c) predicted a maximum protein growth efficiency of 47.4% to occur at 31.2°C, while PPV remained at optimal levels at temperatures between 27.3 and 35°C. The model for temperature and PEV showed the maximum growth efficiency in relation to energy occurred at 30.2°C while the optimal response occurred at a range of temperature between 26.2 and 34.1°C (Fig. 7.1d).

7.4. Discussion

This is the first time that feed intake, growth and growth efficiency data have been modelled for juvenile barramundi (<25 g) over their thermal tolerance range. Barramundi provided an interesting species for these experiments, having a wide thermal tolerance for growth efficiency (Katersky and Carter, 2005), a large geographic distribution as well as being a globally important aquaculture species.

The effect of a wide range of temperature on whole body composition is unclear in the literature. It has been shown that body composition parameters are affected mainly by fish size, diet and life-history stage (Shearer, 1994; Shearer et al., 1994; Koskela et al., 1997; Van Ham et al., 2003). While temperature directly affects the feed intake and growth of fish, it apparently has little direct effect on the composition of the weight gained (Koskela et al., 1997). Some studies have shown that whole-body crude protein was significantly affected by temperature, whilst others have shown that whole body total lipid decreases with temperature (reviewed by Shearer, 1994) and at low temperatures, an increase in total lipid (even with reduced rations) was attributed to low metabolic rate. As the temperature increased, the fish were unable to consume enough energy to maintain maintenance requirements and therefore accumulated progressively less lipid into the whole-body (reviewed by Shearer, 1994).

The wide range of temperatures where feed intake and growth remain high, demonstrate barramundi's ability to efficiently utilise the available dietary nutrients over a wide range of thermal conditions. The optimal range for maximum feed intake was maintained for 2°C above that for SGR. The decrease in growth while feed intake remains maximised can be attributed to metabolic demand (Jobling, 1994). As temperature increases within the thermal tolerance range, metabolism will increase exponentially in small juvenile fish (Jobling, 1997). Jobling (1997) suggested that dissolved oxygen (DO) was possibly the limiting factor in the growth potential of a species at high temperatures due to the inability of the respiratory and circulatory systems inability to deliver oxygen to respiring tissues under high oxygen demand. As fish size increases, routine oxygen consumption will generally increase, however on a weight-specific basis small fish consume more oxygen than their larger conspecifics (reviewed by Jobling, 1994). This relationship has been identified in barramundi and when the effect of temperature was introduced it became apparent that larger fish may be more susceptible to problems associated with low DO than smaller fish due to the higher whole animal routine oxygen consumption (Glennocross and Felsing, 2006). Furthermore, the post-prandial increase in metabolism can be nearly double routine

metabolism (Carter et al., 2001; Katersky et al., 2006) and can remain elevated for long periods of time, depending on temperature and meal size (Carter et al., 2001). The added effect of post-prandial metabolic increase causes a further strain to an already stressed system at high temperatures and may be the determining factor as to whether the fish is able to continue to feed. In the current models, this was evident with the sharp decrease in feed intake at high temperatures. The cumulative effect of increased metabolic demand and decreased oxygen availability consequently limit the growth potential of fish as temperature increases past the optimum temperature range (Jobling, 1997; Koskela et al., 1997).

Under optimal conditions, the PPV and PEV ranged from 36.3-47.4 % and 36.4-43.1%, respectively. These values are similar to PPV and PEV values found for larger barramundi (59 and 176 g), 38 and 43 %, 40 and 39 % at 29°C, respectively (Williams et al., 2006). Maximum growth efficiency is dependant upon a balanced diet providing the appropriate protein to energy ratios and essential amino acids (reviewed by Wilson, 2002). In the present experiments, diet formulation was based upon the protein and energy requirements known to promote optimal growth and it is clear that the barramundi were able to utilise the protein and energy available in the diet and therefore maintain maximum efficiency over a wide range of temperatures. Increasing protein to energy ratios above the dietary requirement, results in increased ammonia excretion and protein synthesis (reviewed by Carter and Houlihan, 2001). Unfortunately increased synthesis does not translate into increased protein retention because synthesised proteins are degraded and catabolized, this in turn increasing energy expenditures and decreasing the amount of energy available for growth (reviewed by Bowen, 1987; Carter and Houlihan, 2001). As protein to energy ratios increase for juvenile barramundi, the relationship with feed conversion and growth rate level off once the optimal ratio (26.7 g CP·MJ GE⁻¹) is reached (reviewed by Boonyaratpalin and Williams, 2002; Glencross, 2006). Furthermore, imbalances in dietary essential amino acids can cause greater oxidation of amino acids and decrease growth efficiency in fish (Williams et al., 2001; Conceicao et al., 2003). This is apparent in a series of experiments where barramundi were fed varying levels of crystalline- and protein-bound amino acids (Williams et al., 2001). In relation to dietary lysine, growth efficiency and lysine retention decreased as lysine concentration increased past the optimal level for both forms of amino acids (Williams et al., 2001).

The magnitude of the range for high growth efficiency (PPV and PEV) was 8°C. These patterns are similar to models which have been developed for Atlantic salmon (Forseth et al., 2001), common wolfish, *Anarhichas lupus* (McCarthy et al., 1998) and European sea bass, *Dicentrarchus labrax* (Person-Le Ruyet et al., 2004) and show that growth efficiency plateaus over a wider range of temperatures. The magnitude of the optimal range was much greater than previously recognized for juvenile barramundi because the entire thermal tolerance range was examined in the current study. This is possibly an adaptation related to the different thermal environments experienced throughout their normal life history (Imsland et al., 1996; Jobling, 1997; Duston et al., 2004). This is especially important for small juvenile barramundi migrating from coastal estuaries to shallow inland rivers at high temperatures in Australia and Southeast Asia. Maintaining high growth efficiency under the wide range of temperatures would be essential for survival a time when rapid growth is occurring. This study also confirms how robust barramundi are for aquaculture development

outside their natural geographic distribution, with efficient culture possible under a wide range of temperatures.

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Table 7.1
Ingredient and chemical composition of experimental diets.

<i>Ingredient composition (g kg⁻¹)</i>	<i>Experiment 1</i>	<i>Experiment 2</i>
Fish meal	730	730
Fish oil	70	70
Starch	119	119
CMC	10	10
Choline chloride	10	10
Phosphorus	10	10
Vitamin C (Stay-C)	20	20
Yb-Oxide	1	1
Vitamin premix ^a	15	15
Mineral premix ^b	15	15
<i>Chemical composition (g kg⁻¹ DM)</i>		
Dry matter (g·kg ⁻¹)	937.0	946.9
Crude protein	504.5	503.5
Total lipid	190.5	182.5
Ash	128.5	150.1
Energy (MJ·kg ⁻¹)	20.3	20.5

^aVitamin premix (mg kg⁻¹): vitamin A (7.50), vitamin D (9.00), calcium D-pantothenate (32.68), nicotinic Acid (15.00), vitamin B-12 (0.015), D-biotin (0.23), folic acid (1.50), thiamin HCL (1.68), pyridoxine HCl (5.49), myo-inositol (450.00), α -cellulose (817.91).

^bMineral premix (mg kg⁻¹): CuSO₄ 5H₂O (35.37), FeSO₄ 7H₂O (544.65), MnSO₄ H₂O (92.28), Na₂SeO₃ (0.99), ZnSO₄ 7H₂O (197.91), KI (2.16), CoSO₄ 7H₂O (14.31), α -cellulose (612.33).

Table 7.2
 Temperature, initial and final wet weight and survival for juvenile barramundi in experiments 1 and 2

	Temperature (°C)		Mean body weight (g)		Survival (%)
	Nominal	Measured	Initial	Final	
<i>Experiment 1</i>					
	21	20.6 ± 0.51	2.4 ± 0.05	3.3 ± 0.11	100 ± 0.0
	24	24.0 ± 0.02	2.7 ± 0.07	4.5 ± 0.18	100 ± 0.0
	27	27.3 ± 0.09	2.7 ± 0.08	12.0 ± 0.50	96.7 ± 1.7
	30	30.1 ± 0.02	3.4 ± 0.09	15.3 ± 0.56	98.3 ± 1.7
	33	32.5 ± 0.04	3.6 ± 0.08	16.2 ± 0.71	95.0 ± 5.0
<i>Experiment 2</i>					
	27	26.98 ± 0.04	4.2 ± 0.13	13.1 ± 0.45	98.3 ± 1.7
	33	33.16 ± 0.13	5.1 ± 0.19	22.5 ± 1.71	96.7 ± 1.7
	36	35.36 ± 0.32	4.5 ± 0.12	20.1 ± 1.76	98.3 ± 1.7
	39	38.86 ± 0.05	5.7 ± 0.17	10.3 ± 0.14	95.0 ± 2.9

Mean ± SEM (n = 3)

Table 7.3
Body composition of juvenile barramundi in experiments 1 and 2.

	Temperature (°C) Nominal	Dry matter (g kg ⁻¹)	Crude protein (g kg ⁻¹ WW)	Total lipid (g kg ⁻¹ WW)	Ash (g kg ⁻¹ WW)	Energy (MJ kg ⁻¹ WW)
<i>Experiment 1</i>						
	21	275.7 ^b ± 2.47	158.0 ^{a,b} ± 2.53	61.6 ± 2.05	39.0 ^{a,b} ± 0.72	5.8 ± 0.15
	24	265.8 ^a ± 2.22	146.0 ^a ± 3.74	54.6 ± 3.57	40.3 ^b ± 0.99	5.5 ± 0.06
	27	271.6 ^{a,b} ± 0.68	159.6 ^{a,b} ± 1.41	58.3 ± 2.77	37.6 ^a ± 0.27	5.6 ± 0.06
	30	270.1 ^{a,b} ± 1.59	160.2 ^{a,b} ± 4.04	56.2 ± 1.43	39.2 ^{a,b} ± 0.42	5.8 ± 0.07
	33	272.9 ^b ± 1.23	162.5 ^b ± 1.31	56.1 ± 1.47	37.6 ^a ± 0.32	5.7 ± 0.03
<i>Experiment 2</i>						
	27	262.5 ^b ± 0.17	154.9 ^b ± 1.01	63.5 ^a ± 1.27	37.8 ^b ± 0.37	5.8 ^{a,b} ± 0.67
	33	275.8 ^a ± 0.14	164.2 ^a ± 1.25	63.1 ^{a,b} ± 2.42	39.6 ^b ± 0.78	5.9 ^a ± 0.95
	36	275.6 ^a ± 0.08	163.8 ^a ± 1.54	64.4 ^a ± 2.96	42.1 ^{a,b} ± 1.31	6.1 ^a ± 0.83
	39	263.0 ^b ± 0.23	147.4 ^c ± 1.29	54.0 ^b ± 2.39	44.2 ^a ± 1.53	5.5 ^b ± 0.93

Mean ± SEM (n = 3). Means with similar or no superscripts were not significantly different (P<0.05) between temperatures.

Table 7.4

Relationships between feed intake (FI), growth (SGR), productive protein value (PPV) and productive energy value (PEV) and temperature (°C) in barramundi.

	$y = a + bT + cT^2$			r^2	$F_{2,6}$	P
	a	b	c			
FI ($\text{g}\cdot\text{d}^{-1}$)	-60.29	4.19	-0.06	0.801	12.06	0.008
SGR ($\%\cdot\text{d}^{-1}$)	-51.69	3.73	-0.06	0.862	18.75	0.003
PPV (%)	-265.05	20.06	-0.32	0.824	14.03	0.005
PEV (%)	-208.46	16.08	-0.26	0.803	12.25	0.008

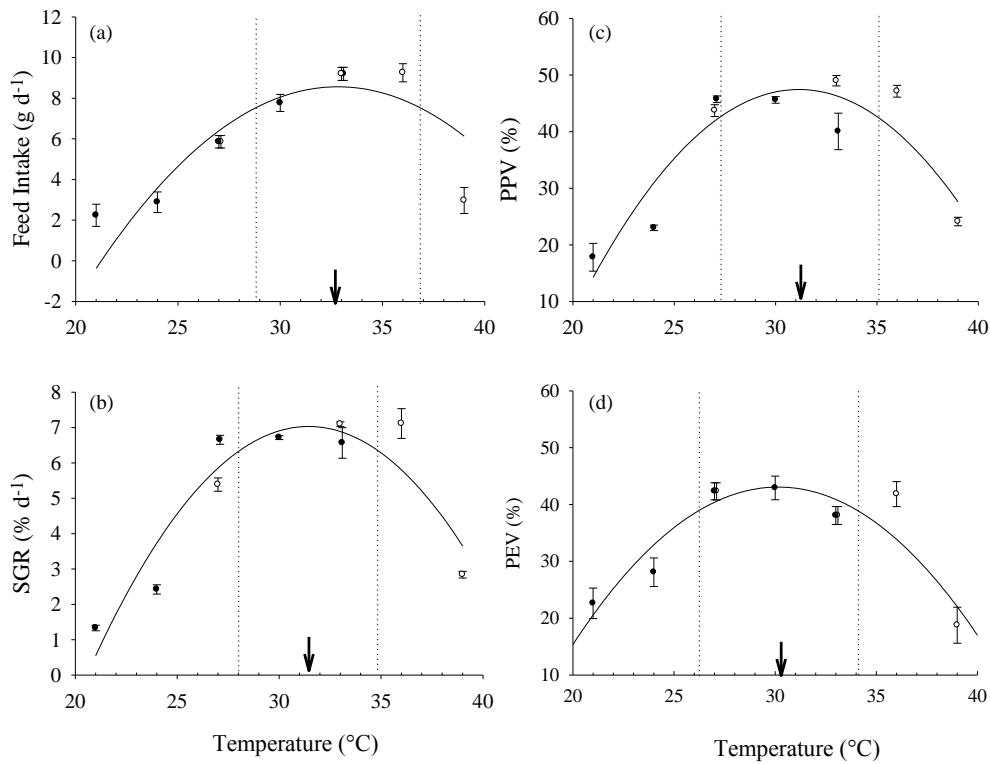


Fig. 7.1.

The relationship between temperature and (a) feed intake (FI, $\text{g}\cdot\text{d}^{-1}$), (b) specific growth rate (SGR, $\%\cdot\text{d}^{-1}$), (c) productive protein value (PPV, %) and (d) productive energy value (PEV, %) for juvenile barramundi. Each relationship is described with a quadratic polynomial. The arrow indicates the temperature where the maximum estimate occurs (optimal temperature) and the vertical lines indicate $\geq 90\%$ of the maximum estimate (optimal range).

Chapter 8*

The effect of temperature on protein synthesis in barramundi, *Lates calcarifer* (Bloch)

Adapted from Katersky, R.S., Carter, C.G. 2007. A preliminary study on growth and protein synthesis of juvenile barramundi, *Lates calcarifer* (Bloch), at different temperatures. *Aquaculture* 267:157-164.

Abstract

Temperature is recognized to be the most important environmental factor affecting growth and protein synthesis in fish. The optimal temperature for growth of juvenile barramundi is 31°C, although culture often occurs at temperatures which are above and below this optimum. Juveniles (2.96 ± 0.46 g) were held at five different temperatures ranging from 21 to 33°C at 3°C intervals. Fish were fed to satiation twice daily (504.5 g kg⁻¹ crude protein, 190.5 g kg⁻¹ lipid, 128.5 g kg⁻¹ ash, 20.2 GE MJ kg⁻¹). Daily feed intake (g), growth (%·d⁻¹), growth efficiency, protein synthesis (measured 24 h after feeding) were determined for each temperature. Feed intake was significantly higher at 33°C, than at any other temperature. Growth and growth efficiency were not significantly different between the 27, 30 and 33°C groups but were significantly higher than the 21 and 24°C groups. In order to take account of variation in protein synthesis over the 24 h following feeding and model daily protein turnover, daily rates of protein synthesis were estimated from previously determined relationships between white muscle and whole body rates of protein synthesis. This showed that protein synthesis was not significantly different between 27 and 33°C and synthesis retention efficiency was over 40% at these temperatures, at 21°C growth efficiency was poor. Growth efficiency and protein metabolism were optimal over a temperature from 27 to 33°C.

8.1. Introduction

Temperature has been identified as the most important abiotic factor affecting growth in ectotherms, including fish (Brett and Groves, 1979). Temperature has a direct effect on feed intake and metabolism, including protein turnover and therefore on the growth efficiency of fish (Jobling, 1994). As temperature increases across the thermal tolerance of a species the feed intake and growth follow asymmetric patterns, gradually increasing to a maximum at the optimal temperature (Jobling, 1997; McCarthy et al., 1999). As temperature increases above the optimum, there is a sharp decrease in both of these parameters and therefore in growth efficiency. Metabolism increases exponentially as temperature increases and at any given temperature, the difference between feed intake and metabolic rate will determine the energy available for growth of the organism (Brett and Groves, 1979; Jobling, 1994). There have been numerous studies and reviews documenting the effects of temperature on growth performance of fish, but few studies have investigated its effects on protein synthesis (McCarthy and Houlihan 1997; Carter and Houlihan, 2001).

Barramundi (*Lates calcarifer*) is a commercially important farmed species in Australia and Southeast Asia and aquaculture has recently expanded to North America and Europe. Production of these fish within Australia has steadily increased for the past 15 years and this trend is expected to continue (Boonyaratpalin and Williams, 2002). The wild fishery for barramundi in northeastern Australia is also a major

industry grossing more than 6 million dollars annually and is surpassed by the recreational fishery. Barramundi are endemic across northern Australia and extend north to Southeast Asia and west to the Persian Gulf. Barramundi have a wide thermal tolerance range (15 to 40°C) and they are commercially cultured at temperatures from 22 to 35°C, however the high and low extreme temperatures which they are cultured at approach the thermal tolerance for this species. Until recently (Katersky and Carter, 2005) studies have not examined temperatures above 30°C.

The influence of temperature on protein synthesis has been measured in some fish (McCarthy and Houlihan, 1997; Carter and Houlihan, 2001), still few studies have concurrently investigated protein synthesis and growth over a temperature range and attempted to relate changes in protein synthesis to the range of temperatures over which growth is optimal. Growth is a reflection of physiological function and therefore protein synthesis is predicted to also exhibit an asymmetric response to temperature. However, in Atlantic wolfish white muscle rates of protein synthesis did not display such a pattern and showed a linear response with temperature (McCarthy et al., 1997). Protein synthesis has only been measured at a limited number of temperatures above the optimal temperature for growth which makes it difficult to determine the temperature response, this is a component of the present study. With barramundi becoming an increasingly important global aquaculture species, this provides an excellent opportunity to gather data on a fish species which is being cultured in a wide range of temperatures. The aims of the present study were to determine the optimal temperature for feed intake, growth efficiency and protein metabolism in juvenile barramundi.

8.2. Materials and methods

8.2.1. Experimental diet

A standard diet was formulated according to known dietary requirements for barramundi (Boonyaratpalin and Williams, 2002). The diet was formulated to contain 50% crude protein and 19.7 MJ kg⁻¹ gross energy (Table 8.1). Fish meal and fish oil were supplied by Skretting (Tasmania, Australia). Vitamins and minerals were supplied by Sigma-Aldrich Pty. Ltd (Sydney, Australia), vitamin C was supplied as Stay-C from Roche Pharmaceuticals (Roche Vitamins Australia Ltd., Sydney, Australia).

8.2.2. Growth experiment

Juvenile barramundi, *Lates calcarifer*, (1-2 g) were obtained from WBA Hatcheries (South Australia, Australia). Fish were maintained at the University of Tasmania under constant environmental conditions (salinity: 10 ‰; photoperiod: 24 h light; temperature: 27°C) and stocked into 5 150⁻¹ aquariums and maintained at 27°C. Temperatures were adjusted 1°C d⁻¹ towards their experimental temperatures of 21, 24, 30 and 33°C, with the exception of the 27°C aquarium that was maintained at a constant temperature. After 6 days all fish were at their experimental temperature. The fish were fed to satiation twice daily for one week at these temperatures. The standard diet was fed to all fish at all times.

At the start of the experiment, 60 fish from each treatment were anesthetized (100 mg l⁻¹, benzocaine) and individual weight (g) and total length (mm) measured. Fish were randomly separated into 3 18-l tanks. Ten fish were euthanised (benzocaine 400 mg l⁻¹) and frozen in liquid nitrogen for whole body chemical composition analysis (see below). Water quality was monitored 3 times week⁻¹ and water changes done as necessary to keep water quality within the limits for barramundi (Tucker et al., 2002). Temperature was recorded hourly with StowAway Tidbit Temperature Loggers (Onset Computer Company, Bourne, MA, USA) and checked manually twice daily.

Fish were fed to satiation twice daily at 0900 and 1800 h for 22 days. A pre-weighed ration was provided to each tank and if completely consumed additional pellets were counted out and provided until feeding ceased. Any uneaten pellets were siphoned out after 10 min and counted in order to determine total daily feed consumption. On day 22, fish from 1 replicate from each treatment were not fed for 24 h and then all individual weight (g) and length (mm) were measured. Two fish from each selected tank were used to measure protein synthesis and 5 fish for whole body composition (see below). On day 23, fish from the remaining 2 replicates of each treatment were not fed for 24 h and sampled as described above.

8.2.3. Protein synthesis

Rates of protein synthesis were measured following a single injection of ³H-phenylalanine using the flooding-dose method (Garlick et al., 1980). Twenty-four h after their last meal, barramundi were anesthetized (benzocaine, 100 mg l⁻¹), weighed and injected with ³H-phenylalanine into the caudal vein at a concentration of 1 ml 100 g body weight⁻¹. The injection solution contained 150 mmol L-phenylalanine and L-[2,6-³H]phenylalanine (Amersham Pharmacia Biotech, NSW, Australia) in 0.2 µm filtered seawater at pH 7.4. The mean measured specific activity of the injection solutions was 1123 ± 118 dpm nmol⁻¹ phenylalanine. Following the injection the fish were returned to separate aquaria containing aerated water (10‰) (Houlihan et al., 1990). Incorporation times varied between 60 and 130 min to investigate the time-course of incorporation to ensure elevated free pool concentrations were stable and incorporation was linear.

Following incubation fish were removed from the tank, euthanised (benzocaine 400 mg l⁻¹) and frozen in liquid nitrogen. The subsequent treatment of samples to measure protein-bound and free-pool phenylalanine-specific radioactivities is as described previously (Houlihan et al., 1986; Houlihan et al., 1990; Houlihan et al., 1995). The fractional rate of protein synthesis (k_s) was calculated using the equations of Garlick et al. (1983). To calculate whole body protein synthesis (WBk_s) the white muscle (WM) free pool specific radioactivity was used as an estimate of the whole body free pool (Carter et al., 1993; Houlihan et al., 1994). Protein consumption rates (k_c , %·d⁻¹) were calculated based on the final protein content of the fish (g protein consumed·g fish protein⁻¹·day⁻¹, Houlihan et al., 1995). Fractional rates of protein growth (k_g , %·d⁻¹) were calculated from the initial and final protein content of fish (Houlihan et al., 1995) at each temperature (see below Table 3 for chemical composition of fish). Fractional rates of protein degradation (k_d , %·d⁻¹) were determined to be the difference between k_s and k_g (Houlihan et al., 1995). Protein concentrations were measured using a modification of the Folin-phenol method (Lowry et al., 1951) and RNA concentrations were measured using dual wavelength absorbance (Ashford and Pain, 1986). RNA was

also expressed as the capacity for protein synthesis (C_s : $\text{mg RNA} \cdot \text{g protein}^{-1}$) and as RNA activity (k_{RNA} , $k_s \cdot \text{g}^{-1} \text{RNA} \cdot \text{d}^{-1}$) (Sugden and Fuller, 1991).

8.2.4. Protein turnover modeling

Daily rates of protein synthesis were estimated by using previously determined relationships between whole body rates of protein synthesis for similar sized juvenile barramundi cultured at temperatures of 21, 27 and 33°C (Katersky, unpublished data). Therefore, protein turnover was only calculated for these three temperatures and data from the 24 and 30°C treatments excluded from further analysis. This model determines the proportion of the 24 h rate of protein synthesis (obtained in the present study) to rates of protein synthesis taken at various times after feeding (Katersky, unpublished data). Mean daily rates of protein synthesis were then calculated. Calculations for protein turnover were according to the formulae used by Houlihan et al. (1995). Synthesis retention efficiency (SRE, %) was determined by dividing the k_g by k_s (Houlihan et al., 1995). Protein retention efficiency (PRE, %) is calculated by dividing k_g by k_c .

8.2.5. Chemical analysis

Standard methods were used to determine dry weight (freeze drying to a constant weight); crude protein (Kjeldahl); total lipid (Bligh and Dyer, 1959); energy (bomb calorimeter); ash by combustion at 550°C for 16h (AOAC, 1995).

8.2.6. Statistical analysis

Data are presented as mean \pm standard error. The normality and homogeneity of data were explored by examining the residual plots. Results were analyzed using a one-way ANOVA (SPSS, version 11.5) and significant results were compared with Tukey's method.

8.3. Results

8.3.1. Growth experiment

Feed intake ($\text{g} \cdot \text{d}^{-1}$) was significantly higher at 33°C than at 27 and 30°C ($F=233.38$; $df=4,10$; $P<0.001$), which were not different from one another, but were significantly greater than the lower temperature groups (Table 8.2). At 33°C feed consumed was $7.63 \pm 0.85 \text{ } \cdot \text{d}^{-1}$, and more than three times that at 21°C. Body weight (g) gain was not significantly different between the 27, 30 and 33°C treatments ($F=71.68$; $df=4,10$; $P<0.001$), however this was significantly greater than at 21 and 24°C. Specific growth rate (SGR, $\% \cdot \text{d}^{-1}$, $F=152.37$; $df=4,10$; $P<0.001$) and growth efficiency (feed efficiency ratio (FER, $F=41.77$; $df=4,10$; $P<0.001$), protein efficiency ratio (PER, $F=41.77$; $df=4,10$; $P<0.001$) followed the same pattern (Table 8.2), there were no significant differences among the high (27, 30 and 33°C) or among the low temperature groups (21 and 24°C) and both groups were significantly different from each other.

At 24°C, whole body crude protein was significantly lower than at 33°C but not significantly different to the other temperature groups ($F=3.77$; $df=4,25$; $P=0.016$, Table 8.3). At 24°C, the ash content was significantly greater than at 27 and 33°C, but

no other significant differences occurred ($F=3.68$; $df=4,25$; $P<0.017$). Crude lipid ($F=1.28$; $df= 4,25$; $P=0.303$) and energy content ($F=2.21$; $df= 4,24$; $P=0.098$) were not significantly different among the 5 temperatures treatments tested (Table 8.3).

8.3.2. Protein synthesis

Validation of the flooding dose method was confirmed from the relationship between the *in vivo* incubation times and the bound and free pools of ^3H -phenylalanine ($\text{dpm}\cdot\text{nmol}^{-1}$ phenylalanine). There was a significant positive linear relationship between the bound ^3H -phenylalanine (s_b) and the *in vivo* incubation time (t) described by, $s_b = 0.007t+0.267$ ($P= 0.048$; $df = 26$; $r^2 =0.15$). The ^3H -phenylalanine free pool (s_a) significantly decreased over the incubation time (t) according to the equation $s_a = -4.656t + 1534.7$ ($P=0.011$; $df = 26$; $r^2=0.23$) and was accounted for in the free pool calculation according to Houlihan et al. (1986).

Fractional rate of protein consumption increased with increasing temperature with the exception of the 27 and 30°C groups ($F=52.95$; $df=4,9$; $P<0.001$, Fig. 8.1d). No significant differences in k_g existed between 27, 30 and 33°C but were significantly greater than 24 and 21°C ($F=204.59$; $df=4,9$; $P<0.001$, Fig. 8.1b). No significant differences in WBk_s were found between temperatures ($F= 2.83$; $df=4,9$; $P=0.090$, Fig. 8.1a). White muscle k_s at 21°C was significantly lower than at 24 and 27°C ($F=6.20$; $df=4,9$; $P=0.011$). There were no significant differences between 24, 27, 30 and 33°C. A consistent trend appeared for all temperatures with the WBk_s being approximately 4 times that of the WMk_s . A significant positive linear relationship exists between WMk_s and WBk_s ($P=0.002$; $df=13$; $r^2=0.57$, Fig. 8.2). Protein retention efficiency was nearly 50% for high temperature groups while the low temperatures retained approximately 20% of the protein consumed. Fractional rates of protein degradation were significantly different between 21 and 24°C and were different from the degradation rates at 27, 30 and 33°C ($F=20.76$; $df=4,9$; $P<0.001$, Fig. 8.1c). Fractional rates of protein degradation at 27, 30 and 33°C were negative, which is not biologically possible.

RNA concentration ($F=2.67$; $df=4,9$; $P=0.102$), C_s ($F=1.06$; $df=4,9$; $P=0.429$) and kRNA ($F=1.07$; $df=4,9$; $P=0.425$) were not significantly different between temperatures and were therefore combined. The mean (\pm S.D.) RNA concentration, C_s and RNA activity were $4.44 \pm 0.34 \mu\text{g}\cdot\text{mg}^{-1}$, $19.37 \pm 2.11 \text{ mg RNA} \cdot \text{g protein}^{-1}$ and $3.10 \pm 0.98 k_s \cdot \text{g}^{-1} \text{ RNA} \cdot \text{d}^{-1}$, respectively (Table 8.4). The relationship between the activity of RNA and whole body protein synthesis is positive and significant ($P<0.001$; $df=13$; $r^2=0.667$, Fig. 8.3). No other relationships between measures of RNA and protein metabolism were significant.

8.3.3. Protein turnover modeling

Daily rates of k_s (mean \pm S.E.) were 11.75 ± 1.35 and $10.34 \pm 1.56 \text{ \%}\cdot\text{d}^{-1}$ for 27 and 33°C, respectively. These rates of synthesis were not significantly different from one another, however they were significantly higher than the k_s at 21°C ($F=9.23$; $df=2,6$; $P<0.015$, Table 8.5). This pattern was the same for k_g ($F=186.71$; $df=2,6$; $P<0.001$, Fig. 8.1) SRE ($F=12.83$; $df=2,6$; $P<0.007$) and PRE ($F=39.17$; $df= 2,6$; $P<0.001$, Table 8.5). Protein degradation ($F=0.65$; $df=2,6$; $P=0.558$) was not significantly different between temperatures (Table 8.5).

8.4. Discussion

The present study is the first to examine feed intake and growth in relation to whole body and tissue protein synthesis in a tropical fish across a wide range of temperatures. It was originally thought these temperatures included the optimal temperature range for juvenile barramundi but we later showed this not to be the case (Katersky and Carter, 2005). The results showed that while feed intake, growth and growth efficiency increased overall as temperature increased, protein synthesis at 24 h after feeding was not different between temperatures of 21-33°C. However, when daily rates of protein synthesis were modeled to take account of variation within 24 h cycles, it was apparent that differences between the temperatures existed.

Previous research has been done on feed intake and growth of juvenile barramundi at different temperatures (Williams and Barlow, 1999), however this is the first study to investigate temperatures above 30°C. The findings in the present study are consistent with results found by Williams and Barlow (1999) where the observed a plateau in FCR between 26 and 29°C. In the present study this plateau continues to 33°C, therefore making it difficult to determine an optimal temperature for feed intake and growth from these data. Additional research has been done by ourselves (Katersky and Carter, 2005) and showed the plateau continues to 36°C.

One aspect of the present study was to examine whether protein synthesis exhibits the asymmetrical response to temperature the way growth does. No study to date has examined a wide enough range of temperature to elicit this response in whole fish. One study has examined this in isolated hepatocytes from rainbow trout (Pannevis and Houlihan, 1992) and found that protein synthesis was maximised near the optimal growth temperature. Differences in protein synthesis in both WM and WB were not significant between the 5 temperatures. This result was surprising due to the large differences in growth between temperatures. Consequently, this analysis produced values for k_d which were negative. The negative results at temperatures of 27°C and above are not biologically possible. This along with the non-significant results of synthesis between temperatures indicated that k_s were underestimated when sampled at 24 h after feeding at temperatures above 27°C. The positive values of protein degradation at 21 and 24°C are most likely reflective of more accurate measures of protein synthesis due to the reduced growth and synthesis rates at lower temperatures (Jobling, 1994; McCarthy et al, 1999).

The relationship between white muscle and whole body protein synthesis is linear regardless of fish species with whole body rates averaging 2-4 times the WM synthesis rates (Houlihan et al., 1988; Carter and Houlihan, 2001). This relationship holds true irrespective of temperature (McCarthy et al., 1999; this study). White muscle has the lowest fractional rates of synthesis of all the major tissues and organs, however because it is the largest protein mass in fish (Carter and Houlihan, 2001), there is considerable interest in using fractional rates of WM synthesis to predict WB synthesis rates. Measuring WM synthesis is much easier due to smaller sample size in comparison to the entire fish (Carter and Houlihan, 2001). Strong relationships between WM and WB protein synthesis have now been demonstrated for rainbow trout (Fauconneau et al., 1990), Atlantic cod (Houlihan et al., 1988), Atlantic wolffish (McCarthy et al., 1999) and barramundi (this study).

A number of studies have examined the relationships between RNA concentration, C_s and k_{RNA} with growth and protein synthesis (Carter et al. 1993; McCarthy et al., 1999) and temperature (Watt et al., 1988; Foster et al., 1992; McCarthy et al., 1999). The results often show that a strong relationship between RNA concentration, protein synthesis and temperature (Carter and Houlihan, 2001) suggesting that fish compensate at suboptimal temperatures by increasing RNA to maintain similar synthesis rates as fish at optimal temperatures (Foster et al., 1992; McCarthy et al., 1999). The present study examined the relationship between these parameters. The relationship between all RNA correlates and growth were not significant (data not shown) and no significant relationship between RNA and temperature existed (Table 4). However, in the present study, variations in protein synthesis rates at all temperatures were explained by an increase in RNA activity (Fig. 4). Increased RNA activity after feeding and not RNA:protein appear to be responsible for the regulation of protein synthesis rates (McMillian and Houlihan, 1989; Mathers et al., 1993).

Measurements of protein turnover are often determined from long-term growth data (weeks to months), short-term protein synthesis measurements (h) and a calculation for protein degradation from the two (Carter and Houlihan, 2001). Single measurements of protein synthesis can be misleading by not accounting for the daily fluctuations which occur. Multiple measurements on the same fish is not possible, however, the measurement from many fish within the same experimental treatment will give a more accurate picture of diurnal rates of protein synthesis including the post-prandial fluctuations which occur (Carter and Houlihan, 2001).

High synthesis retention efficiencies indicate lower protein turnover and therefore more efficient growth in fish (Houlihan et al., 1995). Synthesis retention efficiencies in other species have ranged from 30 to 81% (Houlihan et al., 1995; McCarthy et al., 1999). The SRE found at 27 and 33°C for barramundi fall at the higher end of this range. McCarthy et al. (1999) also found that SRE had an asymmetric response to temperature with maximal SRE occurring at the optimal temperature for growth of Atlantic wolfish. The results of the present study show that SRE as well as the modeled protein synthesis exhibits the same response to temperature as growth with rates plateauing at 27 to 33°C. Unfortunately, due to the optimum temperature not being defined at the time of the present study, temperatures above 33°C were not tested to determine if this asymmetric response occurs in barramundi. The measurement of protein synthesis in barramundi above 33°C is the subject of current research and will provide further data to explore these issues and to further emphasize barramundi as a model species for understanding protein metabolism in tropical fish species.

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Table 8.1
Ingredient and chemical composition of experimental diet

Ingredient composition (g kg ⁻¹)	
Fish meal	730
Fish oil	70
Pre- gelatinized starch	119
CMC	10
Choline chloride	10
Monobasic sodium phosphate (NaH ₂ PO ₄)	10
Vitamin C (Stay-C)	20
Yb ₂ O ₃	1
Vitamin Premix ^a	15
Mineral Premix ^b	15
Chemical composition (g kg ⁻¹ DM)	
Dry matter (g kg ⁻¹)	937.0
Crude protein	504.5
Total lipid	190.5
Ash	128.5
Energy (MJ g ⁻¹)	202.5

^aVitamin Premix (mg kg⁻¹): Vitamin A (7.50), Vitamin D (9.00), Rovimix E50 (150.00), Menadione sodium bisulphate (3.00), Riboflavin (6.00), Calcium D-pantothenate (32.68), Nicotinic Acid (15.00), Vitamin B-12 (0.015), d-biotin (0.23), Folic acid (1.50), Thiamin HCL (1.68), Pyridoxine HCl (5.49), myo-Inositol (450.00), α -cellulose (817.91).

^bMineral Premix (mg kg⁻¹): CuSO₄ 5H₂O (35.37), FeSO₄ 7H₂O (544.65), MnSO₄ H₂O (92.28), Na₂SeO₃ (0.99), ZnSO₄ 7H₂O (197.91), KI (2.16), CoSO₄ 7H₂O (14.31), α -cellulose (612.33).

Table 8.2

Growth, survival, growth efficiency and feed intake of juvenile barramundi at five different temperatures.

Temperature (°C)	21	24	27	30	33
Measured temperature (°C)	20.6±0.51	24.0±0.02	27.3±0.09	30.1±0.02	32.5±0.04
Wet weight _{initial} (g)	2.44±0.05	2.66±0.07	2.68±0.08	3.44±0.09	3.60±0.08
Wet weight _{final} (g)	3.27±0.11	4.54±0.18	11.99±0.50	15.32±0.56	16.18±0.71
Survival (%)	100.00 ± 0.00	100.00 ± 0.00	96.67 ± 1.67	98.33 ± 1.67	95.00 ± 5.00
Feed intake (g·d ⁻¹)	1.25 ± 0.01 ^a	2.05 ± 0.03 ^a	5.62 ± 0.20 ^b	7.50 ± 0.16 ^c	8.66 ± 0.26 ^d
SGR (%·d ⁻¹)	1.27±0.07 ^a	2.32±0.12 ^b	6.37±0.12 ^c	6.42±0.05 ^c	6.28±0.41 ^c
FER (g·g ⁻¹)	0.57±0.05 ^a	0.79±0.05 ^a	1.37±0.02 ^b	1.34±0.05 ^b	1.17±0.09 ^b
PER (g·g ⁻¹)	1.11 ± 0.09 ^a	1.52 ± 0.09 ^a	2.64 ± 0.03 ^b	2.58 ± 0.09 ^b	2.25 ± 0.17 ^b

Mean ± SEM (n = 3). Means with similar superscripts were not significantly different.

Specific growth rate (SGR) = ((lnFBW - lnIBW)/d)*100

Feed efficiency ratio (FER) = BW gain,g (wet)/Mass of food consumed,g (dry)

Protein efficiency ratio (PER) = BW gain (wet)/Mass of Protein fed (dry)

Table 8.3
Body composition of juvenile barramundi at five different temperatures.

	Temperature				
	21	24	27	30	33
Dry matter (g·kg ⁻¹)	275.7 ± 2.47 ^b	265.8 ± 2.22 ^a	271.6 ± 0.68 ^{a,b}	270.1 ± 1.59 ^{a,b}	272.9 ± 1.23 ^b
Crude protein (g·kg ⁻¹ WW)	158.0 ± 2.53 ^{a,b}	146.0 ± 3.74 ^a	159.6 ± 1.41 ^{a,b}	160.2 ± 4.04 ^{a,b}	162.5 ± 1.31 ^b
Total lipid (g·kg ⁻¹ WW)	61.6 ± 2.05	54.6 ± 3.57	58.3 ± 2.77	56.2 ± 1.43	56.1 ± 1.47
Ash (g·kg ⁻¹ WW)	39.0 ± 0.72 ^{a,b}	40.3 ± 0.99 ^b	37.6 ± 0.27 ^a	39.2 ± 0.42 ^{a,b}	37.6 ± 0.32 ^a
Energy (MJ·kg ⁻¹ WW)	5.77 ± 0.15	5.53 ± 0.06	5.62 ± 0.06	5.79 ± 0.07	5.72 ± 0.03

Initial group (mean ± S.D., n=10): Dry matter, 255.9 ± 8.83 g·kg⁻¹, Crude protein, 149.6 ± 1.75 g·kg⁻¹ WW,

Crude lipid, 42.6 ± 1.14 g·kg⁻¹ WW, Ash, 35.6 ± 1.78 g·kg⁻¹ WW, Energy, 5.20 ± 0.12 MJ·kg⁻¹ WW.

(mean ± standard error) Means with similar or no superscripts (P<0.05, n=3) were not significantly different between temperatures.

Table 8.4
 Measurements of RNA of juvenile barramundi at five different temperatures

Temperature	21	24	27	30	33
RNA (mg·mg sample ⁻¹)	4.28 ± 0.13	4.38 ± 0.12	4.89 ± 0.14	4.32 ± 0.06	4.28 ± 0.24
Cs (mg RNA·g protein ⁻¹)	19.94 ± 1.81	19.26 ± 1.02	21.08 ± 1.14	17.74 ± 0.79	18.29 ± 0.88
kRNA (k _s ·g ⁻¹ RNA·d ⁻¹)	2.27 ± 0.54	3.91 ± 0.74	3.14 ± 0.67	3.12 ± 0.21	3.08 ± 0.35
Mean ± SEM					

Table 8.5
 Protein synthesis (k_s), protein degradation (k_d), synthesis retention efficiency (SRE) and protein retention efficiency (PRE) for juvenile barramundi at three temperatures

Temperature	21	27	33
k_s (%·d ⁻¹)	4.61 ± 0.64 ^a	11.75 ± 1.35 ^b	10.34 ± 1.56 ^b
k_d (%·d ⁻¹)	3.34 ± 0.54	4.97 ± 1.30	3.60 ± 1.26
SRE (%)	27.78 ± 2.38 ^a	59.36 ± 7.18 ^b	67.14 ± 6.67 ^b
PRE (%)	16.25 ± 2.33 ^a	49.38 ± 0.55 ^b	43.09 ± 4.24 ^b

Means with similar superscripts were not significantly different ($P < 0.05$, $n=3$)

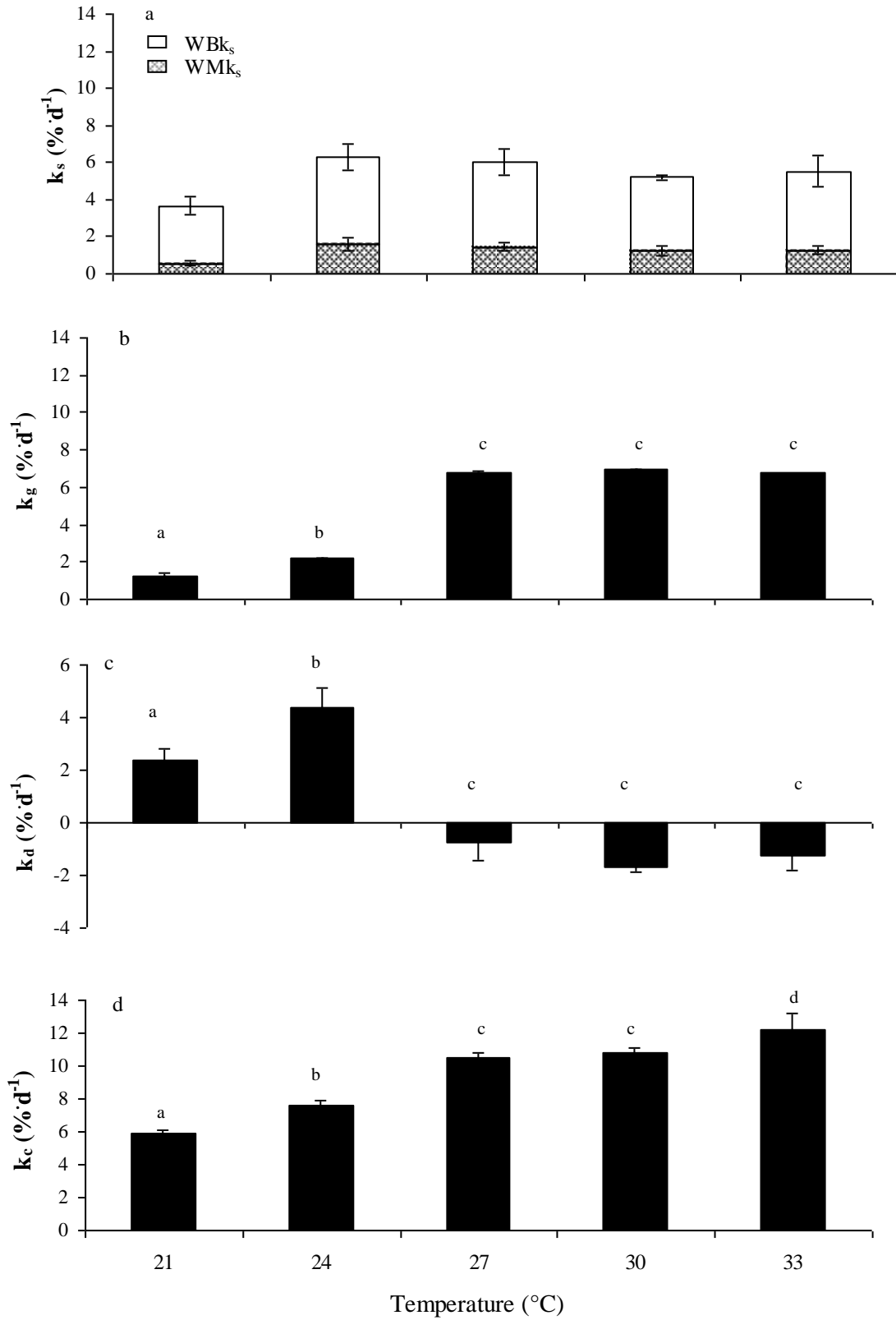


Fig. 8.1. Protein synthesis (k_s , %·d⁻¹,a) for whole body and white muscle, protein growth (k_g , %·d⁻¹, b), protein degradation (k_d , %·d⁻¹,c) and protein consumption (k_c , %·d⁻¹,d) for juvenile barramundi at five different temperatures.

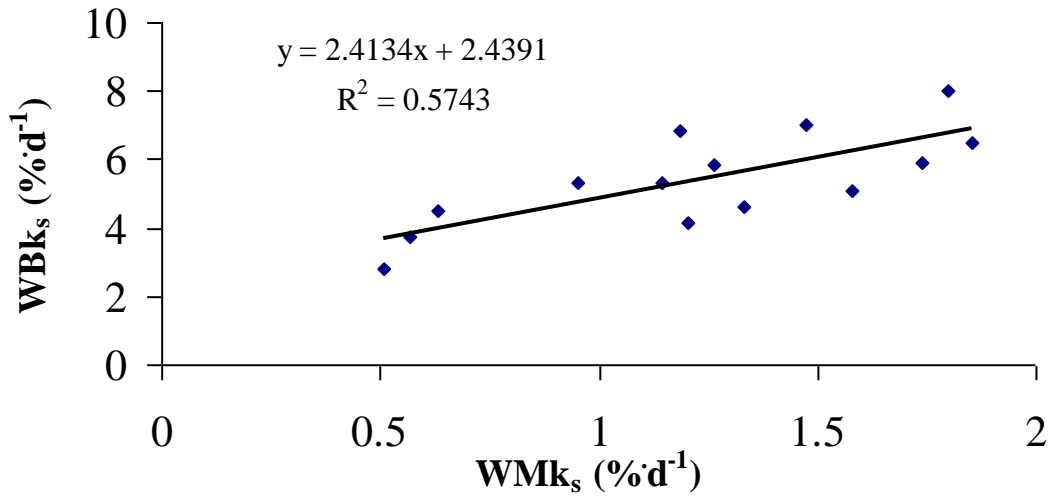


Fig. 8.2. The relationship between white muscle rates of protein synthesis (WMk_s , $\% \cdot d^{-1}$) and whole body rates of protein synthesis (WBk_s , $\% \cdot d^{-1}$) for juvenile barramundi for all temperatures tested.

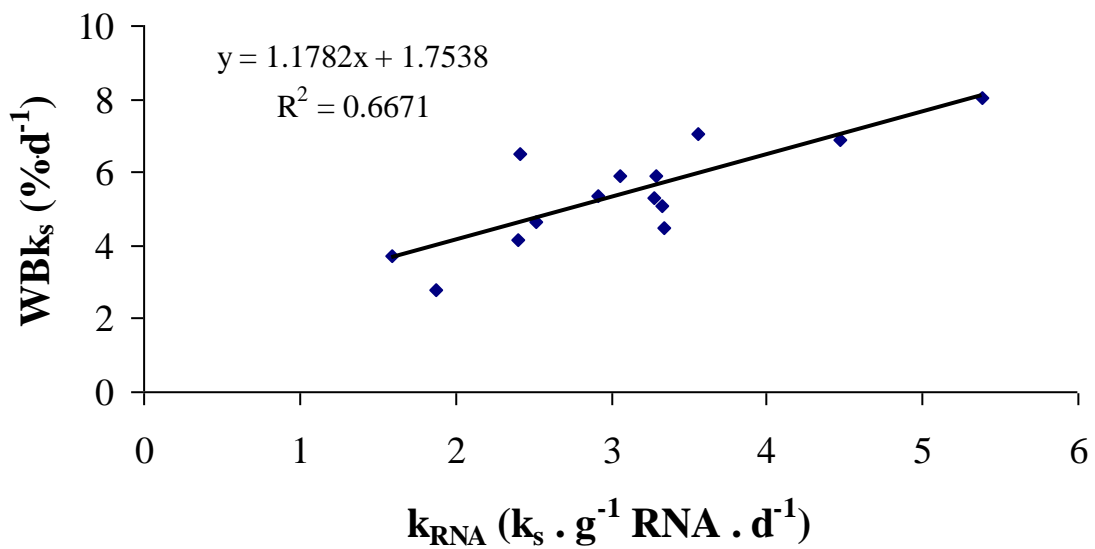


Fig. 8.3. The relationship between RNA activity ($k_s \cdot g^{-1} RNA \cdot d^{-1}$) and whole body rates of protein synthesis (WBk_s , $\% \cdot d^{-1}$) for juvenile barramundi for all temperatures tested.

Chapter 9

Growth performance and protein turnover in barramundi, *Lates calcarifer* (Bloch), fed two dietary protein levels at three temperatures**Abstract**

The experiment aimed to compare the performance of barramundi at optimum and elevated temperatures when fed a standard (OP) and a high protein (HP) diet. In addition, aspects of protein turnover were investigated by measuring changes in the expression of selected genes. Triplicate groups of juvenile barramundi (29-38 g) were held at 29, 33 and 38°C and fed either OP (48% protein) or HP (55% protein). Planned contrasts showed that at 29 and 33°C dietary protein had no effect on barramundi growth performance. At 38°C when fed OP there was a significant decrease in barramundi growth performance compared with both lower temperatures. At 33°C barramundi fed HP had significantly higher whole body dry matter and crude protein. There were few other differences in whole body chemical composition. There were no differences between the 33OP treatment and either 33HP, 38OP or 38HP in the expression of cathepsin D, cathepsin L or β proteasome sub-unit N3. Cathepsin D expression was upregulated at 38°C compared to 33°C, whereas temperature had no effect on the expression of Cathepsin L or β proteasome sub-unit N3. This suggested that cathepsin D was responsive to changes in the nutritional status of the fish. It was hypothesised that at high temperatures the greater energy requirement was met by amino acids supplied by both the diet and increased muscle protein turnover.

9.1. Introduction

Small juvenile barramundi (*Lates calcarifer* (Bloch)) have optimum temperatures in the range 30 to 32°C for growth and growth efficiency (Katersky and Carter, 2007a). The species can be considered eurythermal and maintains high growth and growth efficiency up to temperatures of around 36°C and can still maintain about 50% of maximum growth performance at 39°C (Katersky and Carter, 2007a). This apparently robust ability to deal with high water temperature provides a fish in which to investigate the processes underlying growth and temperature adaptation. Metabolic rate and energy demand increase with increasing temperature (Jobling, 1994), at elevated temperatures the supply of nutrients is likely to be critical and one of the factors limiting growth. In industry, high protein diets are used as “summer” diets and are probably effective partly because they supply an increased amount of protein from which amino acids are used as a more available energy source. Fish muscle also represents a large reserve of amino acids which will be used in periods of nutritional stress. Research on mammals suggests that, quantitatively, the major degradative routes will be via the autophagic-lysosomal and the ubiquitin-proteasome systems, with calpains as a lesser pathway (Attaix et al., 1999). Intriguing differences between protein degradation pathways in fish and mammals are now apparent, for example the proteasome is the major pathway in mammalian muscle but may not be in fish (Martin et al., 2001; Martin et al., 2002; Mommsen, 2004; Salem et al., 2006). A few studies on temperate fish have documented aspects of protein degradation under normal conditions but without integrating to a whole animal level (Martin et al., 2002; Martin et al., 2003; Dobyl et al., 2004; Vilhelmsson et al., 2004).

The experiment aimed to compare the performance of barramundi at low border-line optimum, optimum and elevated temperatures when fed standard and high protein

diets. Aspects of protein turnover were investigated by measuring changes in the expression of selected genes with a focus on cathepsins and the ubiquitin-proteasome system by investigating cathepsins L and D, and β proteasome sub-unit N3 (Chapter 1). Expression of cathepsin D has been proposed as a sensitive marker of protein degradation in fish muscle tissue in relation to different nutritional and environmental conditions. In contrast, cathepsin L and β proteasome sub-unit N3 are not thought to be sensitive to changes in nutritional status and therefore would not be useful markers of muscle protein degradation. Relatively little is known about gene regulation of protein turnover in fish (Fraser and Rogers, 2007), what is known relates to temperature species and the experiment allowed investigation of tropical barramundi.

9.2. Materials and methods

9.2.1. Experimental diets

A standard diet (OP), used elsewhere in this project (Chapters 7 and 8), was compared with a higher protein formulation (HP) in which carbohydrate was replaced with protein (Table 9.1). Fish meal and fish oil were supplied by Skretting Australia (Cambridge, TAS, Australia); pre-gel starch BO11C by Earlee Products, (QLD, Australia); vitamins and minerals by Sigma-Aldrich Pty. Ltd. (Sydney, NSW, Australia); and vitamin C as Stay-C by Roche Vitamins Australia Ltd (Sydney, NSW, Australia). The diets were made as 2 mm pellets on a California pellet mill.

9.2.2. Growth experiment

Juvenile barramundi (0.5-2.0 g) were obtained from WBA Hatcheries (South Australia, Australia) and maintained at the University of Tasmania in 3 150-l aquariums under constant environmental conditions (10‰ salinity, 27°C, 24 h light). Temperatures were adjusted over 10 days by about 1°C d⁻¹ to the experimental temperatures of 29, 33 and 38°C. The fish were fed an equal mixture of the two diets to satiation twice daily for one week at these temperatures.

The fish were then transferred into the experimental recirculation systems which consisted of six identical recirculating systems each consisting of three 18-l carboys with a trickle biofilter on each system. The systems were held at constant temperatures with submersible heaters each controlled by an individual thermostat (Engin and Carter, 2002; Katersky and Carter, 2005). Temperature was recorded every five minutes with StowAway Tidbit Temperature Loggers (Onset Computer Company, Bourne, MA, USA) and checked manually twice daily. During the experiment water quality was monitored 3 times a week and water changes done as necessary to keep water quality within the limits for barramundi (Tucker et al., 2002).

The experiment used triplicate tanks to compare dietary protein and temperature, there were three temperatures of 29, 33 and 38°C and dietary protein of 48 and 55%. At the start of the experiment fish were anesthetized (100 mg L⁻¹, Benzocaine), individual weight and total length measured, and twenty fish returned to each tank. Five fish per tank were euthanised (400 mg L⁻¹, Benzocaine), tissue samples of white muscle removed from 3 fish per tank for analysis of molecular indices of protein turnover (see Chapter 1) and 2 fish per tank were frozen for whole body chemical composition analysis (see below). Fish were fed to satiation twice daily at 0900 and

1800. A pre-weighed ration was provided to each tank and if completely consumed additional pellets were hand fed until feeding ceased. Any uneaten pellets were siphoned out after 10 min and counted in order to determine total daily food intake. On the last day fish were not fed for 24 h and then used to bulk weigh the group. Five fish were removed and sampled as at the start of the trial, individual weight and total length were measured, samples taken for protein degradation and whole body chemical analysis (see below). Samples of white muscle were dissected from 3 fish per treatment and used for analysis of gene expression (Chapter 1).

9.2.3. Chemical analyses

Standard methods were used to determine whole body chemical composition (Carter and Hauler, 2000). Dry matter was measured by freeze drying to constant weight, crude protein by Kjeldahl ($N \times 6.25$), total lipid by chloroform methanol extraction (Bligh and Dyer, 1959). Energy was calculated from protein and lipid content using values of $23.6 \text{ kJ}\cdot\text{g}^{-1}$ protein and $36.2 \text{ kJ}\cdot\text{g}^{-1}$ lipid (Brafield, 1985).

9.2.4. Statistical analysis

Data are presented as the mean \pm SEM (standard error of the means) from 3 replicate tanks. Unfortunately one of the 38°C systems broke and the data from three tanks were removed, this meant 2 tanks remained for OP but only 1 for HP. Consequently, treatment 38HP results were not included in the statistical analysis, but are still presented for visual comparisons. Furthermore, the loss of the system meant it was not possible to examine the interaction between temperature and protein level using 2-way ANOVA. However, the effect of temperature and protein level was assessed separately using a series of orthogonal planned-contrasts (Day and Quinn, 1989). Four contrasts were undertaken: OP diet at 29°C and 38°C ; OP diet at 33°C and 38°C ; OP and HP diets at 29°C ; OP and HP diets at 33°C . Results were analysed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA), and t-tests (SPSS, version 12.0). Growth data were analysed using analysis of covariance (ANCOVA; SPSS, version 12.0) between the initial and final weight measurements as significant differences in mean weight of fish were found between the temperatures at the start of the experiment. Significance was accepted at probabilities of 5% or less.

9.3. Results

9.3.1. Growth performance

Significant differences among initial wet weight did not have a significant effect on final wet weight (ANCOVA). Due to an error in feeding there was a significantly lower feed intake for treatment 33OP compared to the other treatments (Tables 9.2, 9.3). Lower feed intake for 33OP was therefore significant in the interpretation of the planned contrasts for 33OP between 38OP and between 33HP. Therefore, feed intake at 29°C was not influenced by diet where as it was at 33°C (Table 9.2). SGR, FER and PER were significantly higher for 29OP compared to 38OP, and for 33OP compared to 38OP and generally treatment 38OP had the lowest SGR, FER and PER (Table 9.2, 9.3). Overall, diet did not affect SGR, FER or PER at either 29 or 33°C whereas temperature appeared to have an affect. Compared to 38OP the data for 38HP

suggested that the higher protein produced higher performance at the higher temperature.

All measures of whole body chemical composition were affected by diet or temperature and there were significant differences between one or more planned contrast (Tables 9.4, 9.5). Dry matter and crude protein content were significantly higher for 33HP than 33OP, there were no other significant differences. Total lipid was significantly higher for 29OP than 38OP. Protein growth and, protein and energy growth efficiency were affected by temperature but not by diet. Thus, diet OP had significantly lower k_g , $SGR_{EnergyCP}$, $SGR_{EnergyCL}$, PPV, PEV_{CP} and PEV_{CL} at 38°C than at 29 or 33°C (Tables 9.6, 9.7). In contrast, there were no significant differences between OP and HP at 29 or 33°C.

9.3.2. Gene expression

Gene expression was investigated in three fish per treatment, three from each diet and at both 33 and 38°C (this meant fish in treatment 38HP came from the same tank). There were no differences between the OP33 treatment and either 33HP, 38OP or 38HP in the expression of Cathepsin D, Cathepsin L or β proteosome sub-unit N3 (Fig 9.1). There were no diet effects so the two temperatures were compared, there was a significant difference in Cathepsin D between 33 and 38°C, and expression was upregulated at the higher temperature (Fig 9.2). Temperature had no effect on the expression of Cathepsin L or β proteosome sub-unit N3.

9.4. Discussion

The experiment aimed to demonstrate the biochemical mechanisms underlying changes in protein metabolism and growth performance across a range of temperatures including one at the upper extreme. The experiment was designed to control for feed intake and remove differences in feed intake (but not protein intake) as a potential factor in order to investigate the effect of temperature on growth performance and gene expression. Our previous experiments have established satiation feed intake and associated growth (Katersky and Carter, 2007b). Unfortunately the experiment did not work out as planned but the results were both highly original and important in advancing understanding of the physiology of fish at elevated temperature.

The loss of the 38HP treatment meant that diet and temperature effects could not be compared as had been planned. Consequently planned-contrasts were used. Comparisons of 29OP with 29HP and 38OP were done on the basis of equal feed intake in order to investigate the temperature effect. The lower than planned feed intake on 33OP means that comparison of 33OP with 33HP and 38OP involve feed intake as a factor as well as diet or temperature. Whilst 33°C is closer to the optimum temperatures for feed intake and various measures of growth performance 29°C remains within the optimum range, defined as within 90% of maximum performance (Katersky and Carter, 2007b).

Judging by the comparisons at 29°C the high protein diet did not have any advantage for barramundi within the optimum temperature range. Feed intake, growth and growth efficiencies were similar, differences in PPV related to increased protein intake on the HP diet. Visual inspection suggested that the 33HP treatment retained

protein more efficiently than at 29°C, this may reflect the closeness of 33°C to the optimum temperature for maximum PPV (Katersky and Carter, 2007b). Nutrient intake was significantly limiting growth at 38°C, mean wet weight growth was zero: this, the low whole body lipid content and the negative growth efficiency values indicate the fish were using available nutrients to meet their maintenance requirements. Whilst the 33OP fish were underfed and performing more poorly than 33HP they grew and were at a higher nutritional status than 38OP (which ate about 20% more OP diet).

There was clearly no advantage of HP at optimum temperatures. The higher growth and growth efficiency of one tank of barramundi fed the same amount of the HP as two groups fed OP at 38°C argues for the use of high protein diets at high temperatures. This should be investigated further to confirm, or otherwise, our preliminary results. The dietary protein requirement varies between about 45 to 50% (Boonyaratpalin and Williams, 2002), and our previous research (Katersky and Carter, 2007b) with the size of barramundi used in the present study achieved high retention of protein and energy using a 50% DM crude protein diet. The diet used in the present diet contained 55% crude protein and diets with higher protein content should be tested. It should also be noted that the experimental design restricted the feed intake and satiation feeding may reduce the protein requirement (as a proportion of the diet).

Changes in gene expression of cathepsins D and L and for β proteasome sub-unit N3 was compared between 3 fish fed each diet at both 33 and 38°C. PPV reflects the nutritional status of the treatments and can act as a way to consider differences in the experimental history of the fish given the issues discussed above. Mean values were 5, 35, -80 and -46% for 33OP, 33HP, 38OP and 38HP, respectively. The lack of statistical difference is emphasised but the β proteasome sub-unit N3 appeared to be down-regulated for 33HP which showed good growth performance. Activity of the ubiquitin-proteasome complex, measured by 20S proteasome activity in the liver, increased with decreasing rainbow trout growth rate (Dobly et al., 2004). At 33°C the barramundi had positive nutritional status whereas it was negative at 38°C, pooling data showed a significant up-regulation of cathepsin D expression. The involvement of cathepsin D in muscle protein degradation has been measured directly as well as shown by changes in expression (Mommssen, 2004).

This experiment provided an indication that further research is required to determine the protein and energy requirements of barramundi at high temperatures. It also resulted in the characterisation of barramundi genes involved in protein turnover and an indication that they could be used to gain a greater understanding of the nutrition of fish at elevated temperatures. The experiment described here was completed before other parts of the report and informed the approach taken in Chapters 5 and 6.

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Table 9.1
Ingredient and chemical composition of experimental feeds

Ingredient composition (g kg ⁻¹)	OP	HP
Fish meal	730	830
Fish oil	70	59
Pre-gel starch	119	30
CMC	10	10
Vitamin premix ^a	15	15
Mineral premix ^b	15	15
Vitamin C	20	20
Choline chloride	10	10
Monosodium phosphate	10	10
Ytterbium oxide	10	10
Chemical composition (g kg ⁻¹)		
Crude protein	480	545
Total lipid	150	150
Carbohydrate	119	30
Energy (kJ g ⁻¹)	18.9	18.9

^a Vitamin premix (mg kg⁻¹): vitamin A (7.50), vitamin D (9.00), rovimix E50 (150.00), menadione sodium bisulphate (3.00), riboflavin (6.00), calcium D-pantothenate (32.68), Nicotinic Acid (15.00), Vitamin B-12 (0.015), D-biotin (0.23), Folic acid (1.50), Thiamin HCL (1.68), Pyridoxine HCL (5.49), myo-inositol (450.00), α -cellulose (817.91).

^b Mineral premix (mg kg⁻¹): CuSO₄·5H₂O (35.37), FeSO₄·7H₂O (544.65), MnSO₄·H₂O (92.28), Na₂SeO₃ (0.99), ZnSO₄·7H₂O (197.92), KI (2.16), CoSO₄·7H₂O (14.31), α -cellulose (612.33).

Table 9.2

Growth performance of juvenile barramundi at three different water temperatures (29, 33, 38°C) and fed optimum (OP) and high (HP) protein diets

Parameter	Treatments						ANOVA		
	29OP	29HP	33OP	33HP	38OP	38HP ¹	<i>F</i>	df	<i>P</i>
Temperature (°C)	29.09 ± 0.01	29.09 ± 0.01	32.74 ± 0.01	32.74 ± 0.01	38.07 ± 0.00	38.07 ± 0.00	-		
Weight initial (g) ²	0.96 ± 0.12	1.10 ± 0.76	1.37 ± 0.15	1.31 ± 0.21	3.19 ± 1.21	2.33	4.744	4,9	0.025
Weight final (g)	3.24 ± 0.21	3.94 ± 0.30	3.01 ± 0.19	3.63 ± 0.24	2.99 ± 0.06	3.25	3.147	4,9	0.071
Survival (%)	94.61 ± 1.31	81.33 ± 7.42	89.33 ± 1.33	91.26 ± 0.44	94.00 ± 2.00	70.83	2.038	4,9	0.172
Feed intake (g·d ⁻¹)	1.75 ± 0.01	1.75 ± 0.01	1.48 ± 0.11	1.72 ± 0.01	1.77 ± 0.00	1.70	4.674	4,9	0.026
SGR (%·d ⁻¹)	7.26 ± 0.46	7.51 ± 0.23	4.68 ± 0.50	6.12 ± 0.80	0.08 ± 2.23	1.94	10.489	4,9	0.002
FER (g·g ⁻¹)	1.86 ± 0.05	1.85 ± 0.08	1.45 ± 0.17	1.77 ± 0.16	-0.33 ± 0.98	0.09	7.517	4,9	0.006
PER (g·g ⁻¹)	3.64 ± 0.10	3.26 ± 0.14	2.83 ± 0.33	3.13 ± 0.28	-0.64 ± 1.56	0.16	6.959	4,9	0.008

Treatment values are mean ± SEM (n = 3, except n = 2 38OP)

¹, 38HP treatment was not included in statistical analysis

², significant differences accounted for with ANCOVA

Specific growth rate: SGR = (ln wet weight_F – ln wet weight_I)/d*100

Feed efficiency ratio: FER = wet weight gain (g) / total food consumption (g DM)

Protein efficiency ratio: PER = wet weight gain (g)/ total protein consumption (g CP)

Table 9.3

Planned contrasts for growth performance of juvenile barramundi at three different water temperatures (29, 33, 38°C) and fed optimum (OP) and high (HP) protein diets (Table 9.2)

Parameter	Contrast (df)			
	29OP x 38OP	33OP x 38OP	29OP x 29HP	33OP x 33HP
Feed intake	-0.185 (9)	-3.311 (9)**	0.027 (9)	-3.109 (9)*
SGR	5.646 (9)***	3.619 (9)**	-0.075 (9)	-1.271 (9)
FER	4.768 (9)**	3.868 (9)**	0.035 (9)	-0.784 (9)
PER	4.803 (9)**	3.897 (9)**	0.468 (9)	-0.375 (9)

All treatments n = 3 except n = 2 for treatment 38OP (*, P<0.05; **, P<0.01; ***, P<0.001).

Table 9.4

Whole body chemical composition of juvenile barramundi at three different water temperatures (29, 33, 38°C) and fed optimum (OP) and high (HP) protein diets

Parameter	Treatments						ANOVA		
	29OP	29HP	33OP	33HP	38OP	38HP ¹	<i>F</i>	df	<i>P</i>
Dry matter (g·kg ⁻¹)	256.2 ± 1.99	251.7 ± 2.74	246.8 ± 3.15	294.5 ± 12.81	244.3 ± 4.86	243.4	9.361	4,9	0.003
Crude protein (g·kg ⁻¹ WW)	149.4 ± 2.12	147.8 ± 1.88	144.2 ± 2.08	176.5 ± 7.23	141.0 ± 3.54	137.6	5.5	4,9	0.016
Total lipid (g·kg ⁻¹ WW)	63.5 ± 1.87	60.6 ± 3.24	43.1 ± 4.01	54.2 ± 7.20	38.3 ± 5.04	40.3	5.135	4,9	0.020
Ash (g·kg ⁻¹ WW)	32.4 ± 4.47	29.9 ± 3.09	39.1 ± 1.70	47.8 ± 2.93	43.3 ± 0.23	39.1	5.849	4,9	0.013

Initial group (mean ± SD): Dry matter, 228.1 g·kg⁻¹, Crude protein, 271.1 ± 0.47 g·kg⁻¹, Total lipid, 36.3 ± 0.73 g·kg⁻¹ WW, Ash, 35.0 ± 0.54 g·kg⁻¹ WW, Energy_{CP}, 6.40 ± 0.05 MJ·kg⁻¹ WW, Energy_{CL}, 1.31 ± 0.02 MJ·kg⁻¹ WW.

Treatment values are mean ± SEM (n = 3, except n = 2 for 38OP) (*, P<0.05; **, P<0.01; ***, P<0.001).

¹, 38HP treatment not included in statistical analysis.

Energy_{CP}, energy content from crude protein.

Energy_{CL}, energy content from crude lipid.

Table 9.5

Planned contrasts of whole body chemical composition of juvenile barramundi at three different water temperatures (29, 33, 38°C) and fed optimum (OP) and high (HP) protein diets (Table 9.4)

Parameter	Contrast (df)			
	29OP x 38OP	33OP x 38OP	29OP x 29HP	33OP x 33HP
Dry matter	1.141 (9)	0.241 (9)	0.484 (9)	-5.138 (9)**
Crude protein	0.952 (9)	0.314 (9)	-0.070 (9)	-3.953 (9)**
Total lipid	3.562 (9)**	0.677 (9)	0.461 (9)	-1.747 (9)
Ash	-2.285 (9)*	-0.886 (9)	0.576 (9)	-2.055 (9)

All treatments n = 3 except n = 2 for treatment 38OP (*, P<0.05; **, P<0.01; ***, P<0.001).

Table 9.6

Growth rates and efficiencies of juvenile barramundi at three different water temperatures (29, 33, 38°C) and fed optimum (OP) and high (HP) protein diets

Parameter	Treatment						ANOVA		
	29OP	29HP	33OP	33HP	38OP	38HP ¹	F	df	P
k_g (%·d ⁻¹)	3.45 ± 0.48	2.77 ± 0.40	0.43 ± 0.56	3.13 ± 1.05	-4.06 ± 2.29	-3.74	9.406	4,9	0.003
SGR _{EnergyCP} (%·d ⁻¹)	3.44 ± 0.48	2.77 ± 0.40	0.43 ± 0.56	3.13 ± 1.06	-4.06 ± 2.30	-3.74	9.406	4,9	0.003
SGR _{EnergyCL} (%·d ⁻¹)	10.24 ± 0.30	9.26 ± 0.09	5.11 ± 0.08	8.06 ± 1.47	0.02 ± 2.91	0.89	11.674	4,9	0.001
PPV (%)	34.52 ± 2.40	28.18 ± 4.55	5.16 ± 7.50	35.23 ± 10.76	-80.22 ± 56.22	-46.48	6.294	4,9	0.011
PEV _{CP} (%)	34.50 ± 2.79	28.15 ± 4.17	5.13 ± 7.50	35.21 ± 10.76	-80.26 ± 56.24	-46.51	6.31	4,9	0.011
PEV _{CL} (%)	77.98 ± 4.81	78.71 ± 4.67	39.73 ± 3.08	67.52 ± 15.74	-3.89 ± 26.26	7.03	8.007	4,9	0.005

Treatment values are mean ± SEM (n = 3, except n = 2 for 38OP)

¹, 38HP treatment not included in statistical analysis

Fractional protein growth, $k_g = ((\ln P_2 - \ln P_1)/d) \cdot 100$

Specific growth rate for protein energy, $SGR_{EnergyCP} = ((\ln(\text{Crude protein energy}_F) - \ln(\text{Crude protein energy}_I))/d) \cdot 100$

Specific growth rate of non-protein energy, $SGR_{EnergyC:L} = ((\ln(\text{Crude lipid energy}_F) - \ln(\text{Crude lipid energy}_I))/d) \cdot 100$

Productive protein value, $PPV = (\text{fish protein gain (g CP)}/\text{total protein consumed (g CP)}) \cdot 100$

$PEV_{CP} (\%) = (\text{fish Energy}_{CP} \text{ gain (g MJ)}/\text{total Energy}_{CP} \text{ consumed (g MJ)}) \cdot 100$

$PEV_{CL} (\%) = (\text{fish Energy}_{CL} \text{ gain (g MJ)}/\text{total Energy}_{CL} \text{ consumed (g MJ)}) \cdot 100$

Table 9.7

Planned contrasts for growth rate and efficiency measures of juvenile barramundi at three different water temperatures (29, 33, 38°C) and fed optimum (OP) and high (HP) protein diets (Table 9.6)

Parameter	Contrast (df)			
	29OP x 38OP	33OP x 38OP	29OP x 29HP	33OP x 33HP
k_g	5.334 (9)***	3.192 (9)*	0.538 (9)	-2.144 (9)
$SGR_{EnergyCP}$	5.334 (9)***	3.192 (9)*	0.538 (9)	-2.144 (9)
$SGR_{EnergyCL}$	6.080 (9)***	3.024 (9)*	0.654 (9)	-1.963 (9)
PPV	4.347 (9)**	3.235 (9)*	0.269 (9)	-1.274 (9)
PEV_{CP}	4.353 (9)**	3.239 (9)*	0.269 (9)	-1.275 (9)
PEV_{CL}	4.761 (9)**	2.537 (9)*	-0.047 (9)	-1.807 (9)

All treatments n = 3 except n = 2 for treatment 38OP (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

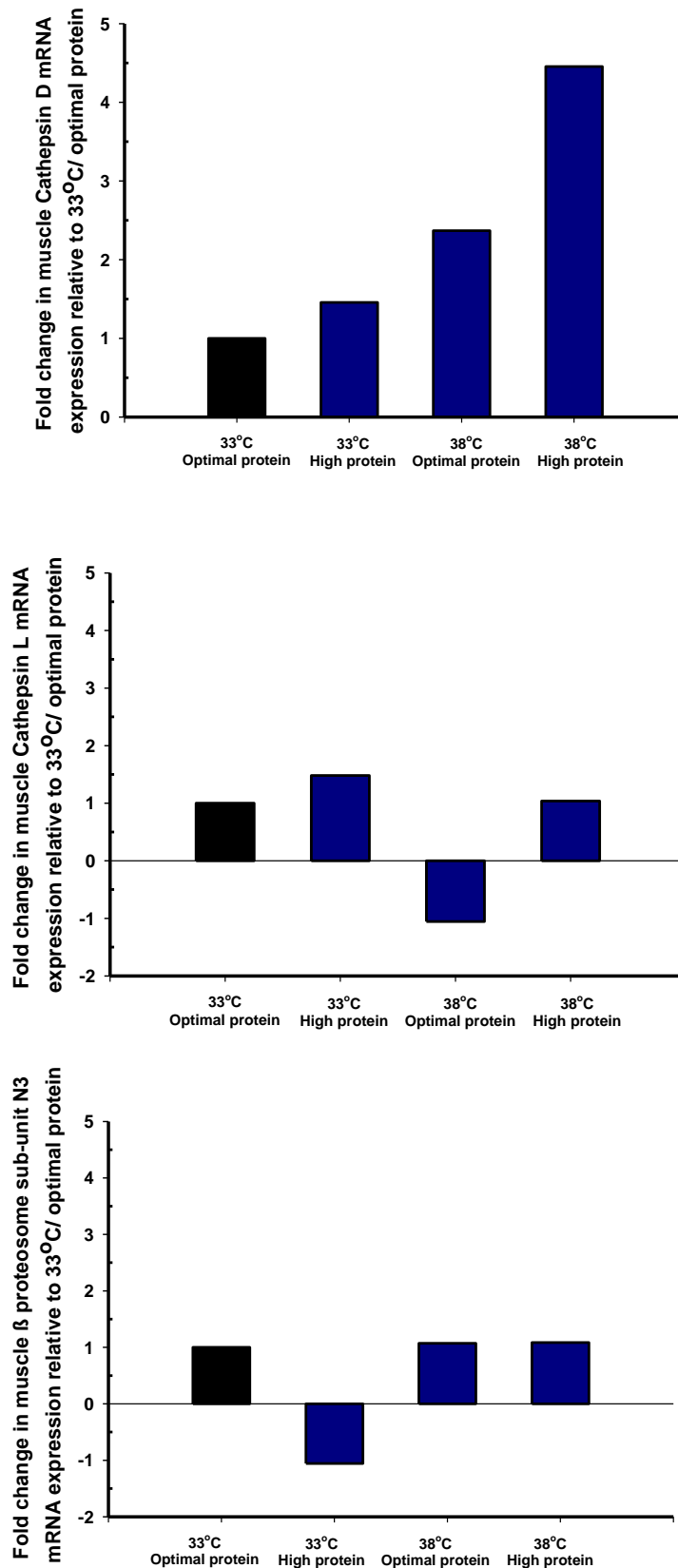


Fig. 9.1. Expression of a) Cathepsin D, b) Cathepsin L, and c) β proteasome sub-unit N3 from juvenile barramundi at three different water temperatures (33, 38°C) and fed optimum (OP) and high (HP) protein diets (n = 3). No significant differences between 33OP and each of the other treatments.

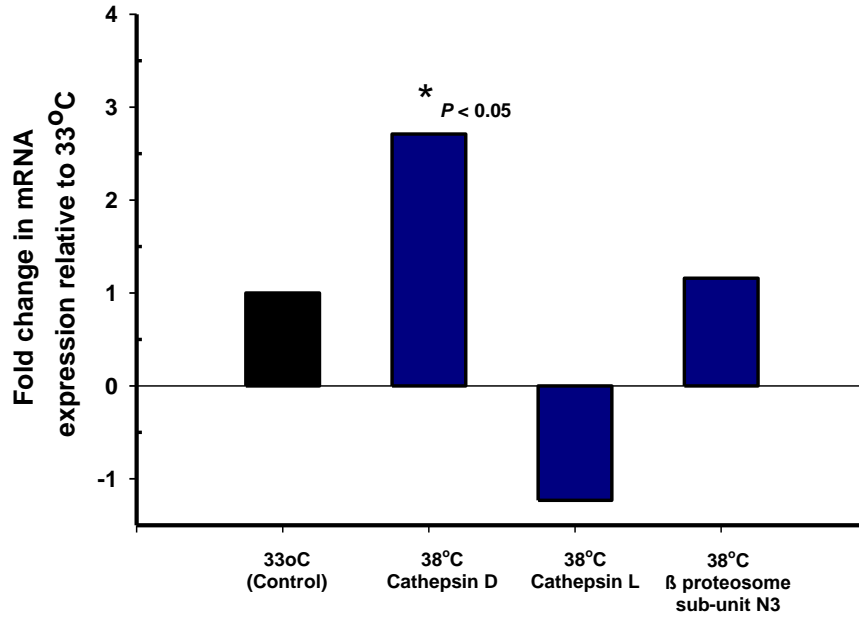


Fig. 9.2. Expression of white muscle Cathepsin D, Cathepsin L and proteasome sub-unit N3 at high temperature treatments relative to control (optimum) temperature. There was no significant dietary effect and diets were combined (n = 6).

Benefits and Adoption

Enhanced capacity in nutrition research through the development of a strength in molecular nutrition at the University of Tasmania. The research for this project concentrated on protein metabolism. Molecular techniques have already been applied to research on fish oil replacement by linking changes in gene expression to replacement oils; to highlight changes in bone formation in relation to high temperature and mineral deficiency; and to understand southern bluefin tuna growth physiology in a FRDC funded project.

Molecular nutrition capacity is strategically placed to contribute further to the salmon and barramundi industries as well as to understanding current issues such as winter syndrome and the effects of low temperature on some marine species.

Skretting, as the commercial partner, has derived benefit from their direct participation in research planning and immediate use of data for feed formulation. The Atlantic salmon industry has derived benefit from greater knowledge of feed manufacturers.

Ridley Aquafeeds have been aware of the project and provided financial support for the presentation of barramundi data at industry conferences.

People development and increasing the capacity in nutrition available to the Australian industry has been increased through training research students: 2 PhD, 1 Masters and 1 Honours were directly involved. Several technicians and students, through casual employment, have gained experimental and technical skills and knowledge from working on the project (Appendix 2).

Findings from the research have been incorporated into undergraduate and postgraduate coursework teaching at UTAS.

The graduation of students, presentation of results at international conferences and publication in peer-reviewed literature contribute to the position of UTAS and Australia having world class aquaculture researchers.

Further Developments

Further developments are expected in relation to the capacity to conduct nutrition research at a molecular level. Current projects will use the methods developed here to investigate mechanisms underlying temperature adaptation in barramundi, optimising ingredients and diets for barramundi and fish oil replacement in Atlantic salmon and barramundi.

The benefits of the research will be greater if on-farm validation is conducted in order to develop an approach that uses changes in specific genes to make rapid assessment of changes in status of fish in relation to environmental, nutritional or farm management factors.

Planned Outcomes

Determination of the optimum dietary protein energy ratio for Atlantic salmon in relation to temperature will benefit the salmonid farming industry, this industry identified the need to know the correct dietary composition to use at higher summer water temperatures; feed manufacturers, this industry needs to have an accurate determination of requirements at different temperatures as a basis for feed formulation.

Accurate description of the maintenance energy and protein requirements of Atlantic salmon. Benefit to the feed manufacturer and salmonid farming industry sectors in providing the basis of differences in the efficiency of diet utilisation.

Biochemical tools that can be used easily on a variety of species and that are shown to relate to complex growth physiology involved in protein turnover, both protein synthesis and protein breakdown, and in digestive function. Tools that can be used to investigate whole-animal and tissue dynamics (particularly muscle and digestive tract). Benefit to other aquaculture industries such as tuna and yellowtail. Tools will allow investigation of the so called "slow growth syndrome" reported for several species.

Detailed understanding of the effects of temperature on protein synthesis and breakdown on the growth of barramundi, as a model of a tropical species at low and high temperatures. Benefit to the barramundi farming sector through understanding the regulation of protein synthesis and breakdown, the mechanism that links nutrient intake to growth, and growth in barramundi at low and high temperatures. Development of biochemical indices will benefit the barramundi farming sector by providing tools for further nutritional research.

Detailed understanding of the effects of temperature on protein synthesis and breakdown and on the growth of salmon, as a model cold-water species at high temperatures. Benefit to the salmonid farming sector through understanding the regulation of protein synthesis and breakdown, the mechanism that links nutrient intake to growth, and growth in salmonids at normal and high temperatures. Development of biochemical indices will benefit the salmonid farming sector by providing tools for further nutritional research.

Conclusions

This research has advanced our understanding of how fish growth is influenced by nutrition, by environment and by the interaction between nutritional and environmental factors. When the research started the majority of nutrition research considered the performance of feeds under optimum environmental conditions. In stark contrast the Australian aquaculture industry is increasingly facing the proposition of growing fish under sub-optimum conditions. The research addressed a significant need for fundamental and applied information about nutrition of fish under limiting environment conditions. Integration of molecular techniques into the research program enhanced the value of this research considerably.

We successfully modelled protein and energy requirements for seawater Atlantic salmon at an elevated temperature of 19°C. Atlantic salmon exhibited the same type of growth response to differences in dietary composition and nutrient intake as at lower

temperatures. The research provided critical and previously unknown information for aquafeed companies to use for their high temperature feed formulations.

A critical feature of elevated temperature is decreased oxygen content in water. A second experiment compared the performance of seawater Atlantic salmon at 19°C at moderately low dissolved oxygen typical of those experienced in summer on-farm. Overall, growth performance was significantly affected by oxygen but only marginally by diet composition. Low oxygen decreased appetite and caused lower growth, growth efficiency was not affected.

Two barramundi growth trials were used to model the effects of temperature on the feed intake and growth performance across a wide temperature range from 21 to 39°C. The optimal temperature for growth of juvenile barramundi was 31°C. Feed intake, SGR and growth efficiency remained at $\geq 90\%$ of the maximum biological response over a large temperature range of 8°C. These models showed that juvenile barramundi are eurythermal and therefore maximise growth by adopting strategies to optimise nutrient utilisation over as wide a temperature range as possible.

An important achievement of the project was to understand potential uses of biochemical and molecular tools in nutrition research. To do this, the utilisation and fates of dietary and body protein were measured in different ways. Protein synthesis was measured in barramundi across a broad temperature range. Protein synthesis was not significantly different over the optimum temperature range and, more critically, synthesis retention efficiency was highest over this range. Protein is expensive to make and it was retained most efficiently during optimum growth. In barramundi cathepsin D was responsive to differences in nutritional status of feeding fish held at an elevated temperature.

In salmon starvation resulted in significantly higher expression of muscle cathepsin L and proteasome β subunit N3. Free pool amino acid concentrations in muscle were measured in relation to temperature and starvation to investigate their potential to highlight limiting nutrients. Starvation had a greater effect on free pool amino acid concentrations than temperature, the essential amino acid lysine had a very low concentration which suggested it may be a limiting amino acid. Importantly, in normally feeding Atlantic salmon at 19°C the dietary amino acids were not limiting at this elevated temperature.

By combining molecular and biochemical approaches, a mechanism that fish use to survive high temperatures was proposed: the greater energy requirement at elevated temperatures was met by amino acids supplied by both the diet and muscle protein breakdown. Specific pathways were identified. This explained why high protein diets work at high temperatures.

Appendices

Appendix 1: Intellectual property

Not applicable

Appendix 2: Staff involved

At the time of the research all staff were members of the School of Aquaculture, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania. They were:

Professor Chris Carter

Dr Andrew Bridle

Dr Melanie Leef

Dr Robin Katersky

Mr Keith Irwin

Mr James Dunn

Mr Torben Louwen-Skovdam

Appendix 3

Presentations

- Barnes, JC, Hauler, RC, Carter, CG, 2006. 'The effect of protein: energy ratio on the growth and protein synthesis of Atlantic salmon (*Salmo salar*) at 19°C', *XII International Symposium Fish Nutrition & Feeding*, Biarritz, France.
- Carter, CG, 2004. 'Product evaluation update - Atlantic salmon (Summer Salmon)', *Seeding a Future for Grains in Aquaculture Feeds - Part II. Proceedings of a Workshop*, Fremantle, Western Australia.
- Carter, CG. 2007. 'Nutrition, temperature and lupins'. *FRDC Salmon Aquaculture Sub-program R&D Seminar*, MRL, Tarooma.
- Carter, CG & Bridle, AR 2006. "Nutrition: development of approaches and molecular tools". *FRDC Salmon Aquaculture Sub-program R&D Seminar*, 6th December, CSIRO, Hobart.
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- Carter, CG, Bridle, AR, Katersky, RS, 2006. 'Nutrition, thermal stress, protein turnover and gene expression in fish', *Australasian Aquaculture Conference*, Adelaide.
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- Katersky, RS, Carter, CG, 2006. 'The effect of temperature on protein turnover and growth in juvenile barramundi, *Lates calcarifer*', *XII International Symposium Fish Nutrition & Feeding*, Biarritz, France.
- Katersky, RS, Bridle, AR & Carter, CG. 2007. 'The interaction of temperature and nutrition on protein turnover and gene expression in barramundi, *Lates calcarifer*'. *Australian Barramundi and Prawn Farmers Conference*, Brisbane, 25-27 July. *Invited presentation*
- Katersky, RS, Bridle, AR, Carter, CG, 2007. 'The interaction of temperature and nutrition on protein turnover and gene expression in Asian sea bass, *Lates calcarifer*', *Second International Symposium on the Environmental Physiology of Ectotherms and Plants*, 1-6th July, Dunedin, New Zealand.
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